FURTHER STUDIES ON THE IDENTITY OF PRECIPITINS AND PROTEIN SENSITIZERS (ALBUMINOLYSINS).*

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In a preceding paper (1) the writer reported experiments in which he studied the relation between the alexin fixation by sensitized cells and the same phenomenon as it occurs in connection with the precipitin reaction.

Gengou (2), in his first discovery of alexin fixation by mixtures of proteins with their specific antisera, assumed that in such cases there were formed not only specific precipitins, but albuminolysins, or protein sensitizers, as well. A similar opinion is expressed by Nicolle (3), and Gay (4), in a recent paper, agrees with this view. The presumption in such an interpretation is that the protein-sensitizer complex is brought down mechanically with the precipitate, the consequence being a simulation of alexin fixation by the precipitate itself. Nicolle goes so far as to speak of the "*coagulins*" as "*anticorps bons*," which oppose the action of the lysins, or sensitizers, upon the antigen, thereby preventing the harmful liberation of poisonous products.

Ehrlich (5) says in this connection: ". . . it seems reasonable to assume, in accordance with Gengou's first explanations, that the property of binding the complement is exercised by the albuminous bodies sensitized with a specific amboceptor, and . . . just as when immunizing with cells agglutinins and amboceptors are formed, so also when immunizing with dissolved albuminous bodies, two kinds of antibodies are formed, precipitins and amboceptors."

In our former work on the problem of alexin fixation (6) we found that there was a distinct difference between alexin fixation that occurred when a dissolved protein was mixed with a specific antiserum, and that which followed the mixture of a bacterial filtrate with its antibacterial serum. It was found that, in the former case (sheep serum, anti-sheep serum), the alexin fixation was usually inherent in the precipitate only and absent from the supernatant liquid, except in mixtures in which the mutual propor-

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tions of antigen and antibody were such that precipitation was markedly inhibited.

In contrast to these well known conditions, it appeared that when typhoid filtrate was added to antityphoid serum, alexin fixation was exerted by both the precipitate and the supernatant fluid, even when the relative concentrations of antigen and antibody were at the optimum for precipitate formation. In no case was the supernatant fluid of such mixtures free from alexin-fixing properties far exceeding those of the precipitate. These facts have been confirmed since the former article was written. It appeared to us that this indicated a fundamental difference which made it unlikely that the fixation properties of the precipitate were merely the expression of the mechanical involvement of a sensitizer in the precipitate. It seemed to show that immunization with a cellular antigen incited the formation of both a precipitating and a non-precipitating antibody, both of which formed alexin-fixing complexes when united to their respective antigens, while immunization with an unformed protein gave rise to the precipitating antibody only. And it seems indeed reasonable to suppose that a formed cell might contain structural elements antigenically distinct, and not present in dissolved unformed proteins.

In attempting to prove that the alexin fixation of the precipitates was a phenomenon *per se* and not due merely to the adsorption of cytolytic sensitizer in the precipitate, we found that there was no quantitative diminution of the total fixing power in the supernatant fluid of a precipitated mixture of typhoid filtrate and typhoid antiserum after removal of the precipitate, as compared with that of the same amount of the serum in the presence of whole washed bacteria. We are, after much further study, inclined to lay less stress upon this phase of the experiments since accurate quantitative measurements of this kind are frequently complicated by the slight fixing power inherent in the bacterial cells in the entire absence of serum. However, the belief that the alexin fixation by a precipitate is not due to such mechanical inclusion or adsorption of sensitizer seems still justified. For in the one case (the bacterial filtrate), whatever the proportion of antigen and antibody used, there was always distinct fixation by the precipitate and strong fixa-

tion by the supernatant fluid, while in the other (the unformed antigen) the fixing power resided, as a rule, entirely in the precipitate, even in mixtures in which the proportions of antigen and antibody were so gauged that the precipitate formed was but a slight one. Cases like the following illustrate our meaning.

SHEEP SERUM—ANTI-SHEEP SERUM.

1. Antigen 1 : 10. Heavy precipitate; strong fixation by washed precipitate; slight fixation by supernatant fluid.

2. Antigen 1 : 100. Heavy precipitate; fixation by washed precipitate; none by supernatant fluid.

3 to 6. Antigen dilutions ranging from 1 : 200 to 1 : 5,000. In all cases, although the precipitate is extremely slight in the last tubes, the fixing power resides entirely in the precipitate and not at all in the supernatant fluid.

Such relations are not invariable, but occur with great frequency. In such cases, if the process were merely a mechanical carrying down of sensitizer, one would certainly be justified in expecting larger amounts of alexin-fixing substances to remain in the supernatant fluid as the bulk of precipitate is diminished. As a matter of fact this occurs only when the antigen has been diminished to such a degree that no precipitate or almost none is formed, or in cases, as above in the 1 to 10 dilution of the antigen, where an excess of antigen was used.

It has seemed to us therefore that the simplest conception of alexin fixation by precipitates would be the following: During immunization with a dissolved, unformed protein, sensitizers are formed which render the antigen amenable to the action of the alexin. Since the reaction is one between two mutually precipitable colloids, they precipitate each other, but, in keeping with other reactions between substances of this nature, they do so only when their relative concentrations are within certain zones of proportion which favor precipitation. This constitutes the visible precipitation which may be conceived as merely secondary, and due to the colloidal nature of the reacting bodies, the essential feature of the phenomena being the union of a specific sensitizer with its antigen.

And the assumption that in structure and function the precipitins are to be looked upon merely as protein sensitizers is strengthened by the discovery of Friedberger (7) that the action of alexin upon

a precipitate results in the liberation of anaphylatoxins, just as this occurs when alexin acts upon sensitized cells. An apparent objection to this point of view may be found in the fact demonstrated by Friedberger, Dean, and others, that the optimum of precipitation may not correspond to the optimum for alexin fixation in mixtures of antigen and antibody. This, however, does not seem to us to argue against the conception outlined above, since the basis of this consists in the separation of the process of antigen-antibody union from that of precipitation, the latter being merely secondary and incidental, and governed, as we know, by many fortuitous circumstances, such as relative quantities, and possibly the presence or absence of colloidal protective substances. A strong support for this point of view, indeed, seems to be the fact noted by Kraus and Joachim (8) that heating a precipitin to 60° C. may destroy its precipitating power without interfering with its power of uniting with the antigen. This is analogous to the observation of Porges (9) that heated serum will protect mastic suspensions against precipitation, while unheated serum will precipitate it. And the parallelism between serum precipitation and that of other colloids has been further emphasized by Young (10) and the writer.

The lack of parallelism between alexin fixation and precipitation which is often observed would, therefore, seem rather to strengthen than to detract from the probability of the conception just outlined: an antigen and its antibody react; being colloids they are precipitated when the environment and proportions under which the reaction takes place favor flocculation.

Since it seemed to us, therefore, that a careful consideration of all the facts ascertained rendered it extremely unlikely that alexin fixation by a precipitate was due to the mechanical involvement in the precipitate of a separate antibody, and that there was at least great likelihood that the precipitin itself represented the protein sensitizer, it still remained to show that such fixation was not merely a physical adsorption of alexin by an indifferent suspension such as that which Landsteiner and Stankovíc (11) have described for kaolin, and Seligmann (12) for mastic and salt mixtures.

We were at a loss to find a method of approaching this question until it occurred to us that it might be possible to demonstrate a

similarity between the mechanism of alexin absorption by precipitates and that which is exerted by sensitized cells, after alexin had been divided into the so called end-piece and mid-piece, by the methods of Ferrata (13), Sachs (14), or that of Liefman (15). Experiments were consequently carried out with this in view. The alexin was fractioned by various methods and carefully titrated before every experiment. A determination was made of the inactivity of both end-piece and mid-piece alone, in the presence of sensitized cells; their activity when added to such cells together in proper proportions was ascertained, as was also the hemolytic power of the end-piece when added to sensitized cells previously treated with mid-piece (persensitized cells).

The following is an example of one of our experiments.

A.

PRELIMINARY TITRATION.

1. Sensitized cells + (mid-piece) globulin fraction 0.2 c.c. = 0.
2. Sensitized cells + (end-piece) albumin fraction 0.2 c.c. = 0.
3. Sensitized cells + mid-piece and end-piece (same proportions). Hemolysis in 10 min.
4. Sensitized cells + whole complement 0.1 c.c. Hemolysis in 2 min.
5. Sensitized cells + whole complement 0.05 c.c. Hemolysis in 5 min.

The hemolysis in the tubes in which end-piece and mid-piece were combined was rarely as rapid or complete as with the original complement in proportionate amount.

In the preliminary titrations the alexin fractions were often prepared both by the Ferrata method of dialysis and the method of dilution with N/300 hydrochloric acid, as advised by Sachs and Altmann. Since it was found in experiments carried out in this laboratory that by the Ferrata method it was often difficult to obtain the so called mid-piece (precipitated fraction) free of end-piece without further repeated resolution and precipitation, and that by the Sachs method the supernatant end-piece was often difficult to free of mid-piece,[1] we occasionally combined the Ferrata supernatant fluid with the Sachs precipitate with good results. The experiments were done repeatedly, with a variety of divided alexins, always controlled by titration as above.

[1] These experiments will be reported by Mr. Maltaner, of this laboratory, in a later communication.

B.

The serum-antiserum precipitates were prepared by adding two cubic centimeters of a potent anti-sheep serum precipitating serum (titre 1 to 5,000) to ten cubic centimeters of a 1 to 50 dilution of sheep serum. After two to three hours, in one case twelve hours, the heavy precipitate was centrifugalized, once washed in salt solution, and distributed to the tubes of the experiment as follows:

A. Precipitate + albumin fraction (end-piece).
B. Precipitate + globulin fraction (mid-piece).
C. Precipitate + globulin fraction (mid-piece) + albumin fraction (end-piece).
D. Precipitate + whole complement 0.05 c.c.

In two other tubes the following mixtures were made:

E. Sensitized cells 3 c.c. + mid-piece. This tube was made to furnish persensitized cells for the second step of the experiment and also constituted a further control for the inactivity of the mid-piece alone.

F. Sensitized cells 1 c.c. + end-piece. This tube further controlled the inactivity of the end-piece alone at the time of carrying out the tests, and was used, in the second phase, to demonstrate the inability of the sensitized cells to bind the end-piece alone, furnishing a parallel to the similar tube in which the precipitate was used instead of sensitized cells.

The tubes were kept in a water bath at 40° C. for forty-five minutes. At the end of this time they were centrifugalized and treated as follows:

A′. To the supernatant fluid from tube A were added persensitized cells from the sediment of tube E. Hemolysis in 8 minutes.

B′. To the supernatant fluid of B were added end-piece and sensitized cells. The end-piece was further controlled at this time. No hemolysis after 2 hours at 40° C.; over night at room temperature.

C′. To the supernatant fluid of C were added sensitized cells. No hemolysis, as in B′.

D′. To the supernatant fluid of D were added sensitized cells. Very slight, almost no hemolysis by the next morning. The sensitized cells here used were saturated with sensitizer and amenable to the action of very small quantities of complement.

The experiments show that the precipitate fixes alexin by the same mechanism by which it is accomplished in the case of sensitized blood or bacterial cells. The precipitate fixes alexin as a whole. When fractionation is carried out in the manner indicated, however, it is found that the albumin fraction (end-piece) is not

fixed directly and remains free in the presence of the precipitate unless mid-piece is added with it. The latter, however, is fixed directly, and is independent of the presence of end-piece.

The results, it seemed to us, possessed theoretical significance in favor of the conception of precipitins as sensitizers. It was still possible, however, that all alexin fixation might take place in this way, even that exerted by such indifferent suspensions as kaolin, chalk, quartz sand, etc. Although unlikely, it is not impossible that in such alexin adsorption, the mid-piece must be adsorbed as a necessary step preceding the adsorption of the end-piece. For this reason experiments with this point in view were carried out with kaolin.

About 1.5 gm. of kaolin were ground up in a mortar, at first dry, then with the addition of a little salt solution. This was continued for about ten minutes and the finely ground material was then suspended in 20 c.c. of physiological salt solution. Further dilution of this with salt solution gave a milky suspension which on standing settled out slowly.

1 c.c. of this suspension was found to fix completely 0.05 c.c. of fresh guinea pig alexin.

Guinea pig alexin was then fractionated by the Ferrata method and titrated as follows: the alterations in volume taking place in the course of the fractionation were such that 0.13 c.c. of the albumin fraction (end-piece) and 0.1 c.c. of the globulin fraction corresponded to 0.1 c.c. of the original complement.

The following experiment was then carried out:

1. Kaolin suspension 1 c.c. + complement 0.05 c.c.
2. Kaolin suspension 1 c.c. + albumin fraction (end-piece) 0.13 c.c.
3. Kaolin suspension 1 c.c. + globulin fraction (mid-piece) 0.1 c.c.
4. Kaolin suspension 1 c.c. + globulin fraction 0.1 c.c. + albumin fraction 0.13 c.c.
5. Salt solution 1 c.c. + globulin fraction (mid-piece) 0.1 c.c.

The tubes were kept in a water bath at 40° C. for forty-five minutes. At the end of this time the tubes were centrifugalized and the supernatant liquids carefully removed with pipettes. To these supernatant fluids the following additions were then made:

1′. Supernatant fluid of tube 1 + sensitized sheep corpuscles. No hemolysis.
2′. Supernatant fluid of tube 2 + mid-piece and sensitized corpuscles. No hemolysis.
3′. Supernatant fluid of tube 3 + end-piece and sensitized cells. No hemolysis.
4′. Supernatant fluid of tube 4 + sensitized corpuscles. No hemolysis.
5′. This tube was used as a control to make sure that the mid-piece which

is very unstable in salt solution would not be sufficiently weakened during the time of first exposure to lead to error. To this tube were added end-piece and sensitized cells. Hemolysis in ten minutes.

Final readings were made on the following morning.

From experiments like the foregoing the conclusion can be drawn that in the case of the indifferent suspension kaolin, the fixation of alexin or complement does not take place by the same mechanism by which this is accomplished both in the case of sensitized cells and in that of specific serum precipitates. The kaolin suspension fixes whole complement mid-piece alone, end-piece alone without the presence of mid-piece, and the two fractions together. This fact demonstrates a fundamental difference between fixations of this category and those which are exerted by specific antigen-antibody complexes.

CONCLUSIONS.

The experiments here recorded, as well as those preceding them and outlined in the introductory paragraphs, have had the purpose of analyzing the phenomenon of alexin fixation occurring when dissolved, unformed proteins are added to their specific antisera. The present experiments have shown that specific precipitates have the same relation to the complement fractions first described by Ferrata that are possessed by sensitized cells. In this they differ from indifferent suspensions, like kaolin, which fix alexin and its fractions indiscriminately, fixing the end-piece without dependence upon previous adsorption of the mid-piece.

The writer believes this to be of theoretical importance since it seems to show, in the first place, that the fixation of alexin by precipitates is not merely a mechanical adsorption, and in that it renders more likely the supposition that the so called precipitin is actually a protein sensitizer by which a foreign protein is rendered amenable to the proteolytic action of the alexin. The visible precipitation in such reactions is merely secondary, occurring because of the colloidal nature of the reacting bodies, under conditions of quantitative proportions and environment which favor flocculation. It does not seem necessary to assume a structure for the so called precipitins essentially different from that of other sensitizers.

Carried to its logical consequences, the acceptance of this view,

taking the identity of agglutinins and precipitins at least as a possibility, leads to the conception that functionally there is but one variety of specific antibodies, and that is the sensitizer which makes possible the action of alexin or complement upon various antigens.[2]

BIBLIOGRAPHY.

1. Zinsser, H., *Jour. Exper. Med.*, 1912, xv, 529.
2. Gengou, *Ann. de l'Inst. Pasteur*, 1902, xvi, 734.
3. Nicolle, M., *Compt. rend. Soc. de biol.*, 1907, lxiii, 77; this article is reviewed in *Bull. de l'Inst. Pasteur*, 1907, v, 839.
4. Gay, F. P., *University of California Publications in Pathology*, 1912, ii, 1.
5. Ehrlich, P., Studies in Immunity, translated by Bolduan, C., 2d edition, New York and London, 1910, 585.
6. Zinsser, H., *loc. cit.*
7. Friedberger, E., and Vallardi, C., *Ztschr. f. Immunitätsforsch., Orig.*, 1910, vii, 94.
8. Kraus, R., and Joachim, J., *Centralbl. f. Bakteriol., 1te Abt., Orig.*, 1904, xxxvii, 73.
9. Porges, O., in Kraus and Levaditi, Handbuch der Technik und Methodik der Immunitätsforschung, Jena, 1909, ii, 1146.
10. Zinsser, H., and Young, S. W., *Jour. Exper. Med.*, 1913, xvii, 396.
11. Landsteiner, K., and Stankovic, R., *Centralbl. f. Bakteriol., Orig.*, 1906, xlii, 353.
12. Seligmann, E., *Berl. klin. Wchnschr.*, 1907, xliv, 1013.
13. Ferrata, A., *Berl. klin. Wchnschr.*, 1907, xliv, 366.
14. Sachs, H., in Kraus and Levaditi, Handbuch der Technik und Methodik der Immunitätsforschung, Jena, 1909, ii, 969.
15. Liefman, H., and Cohn, M., *Ztschr. f. Immunitätsforsch., Orig.*, 1910, vii, 669.

[2] Since this article has been in press a piece of work by Skwirsky (Skwirsky, P., *Ztschr. f. Immunitätsforsch., Orig.*, 1910, v, 538) has come to my attention. Skwirsky's work deals also with complement fixation by precipitates, and reaches conclusions in many ways similar to my own.

THE TOTAL NON-PROTEIN NITROGEN OF THE BLOOD IN NEPHRITIS AND ALLIED CONDITIONS.*

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In 1836 Richard Bright,[1] referring to renal disease, stated: "The chemical qualities of the blood are so far changed that urea is to be detected in that fluid, or, at all events, certain constituents scarcely distinguishable from it"; and he expressed the opinion that the occurrence is "a source of disease springing immediately out of the defective action of the kidney." Seventy years later, Friedrich Müller[2] emphasized the fact that "insufficiency of nitrogenous excretion leads to a definite increase in the retention nitrogen of the blood, particularly in those groups of kidney disease in which experience has shown the danger of uremia." These statements embody all that can be asserted with positiveness at the present moment. The attempt to find an invariable relation between the retained nitrogen of the blood and the degree of uremic intoxication has not been successful. On this account, and because the methods in use until recently required considerable quantities of blood, at least five times as much as the procedures we have employed, the determination of the nitrogen or urea of the blood has not become a routine means of studying the efficiency of the renal function.

Strauss[3] and others, a decade ago, tried to connote nitrogen retention with definite pathological types of nephritis, but without success. Widal[4] has viewed the subject from a different angle and has defined several distinct types of disturbed renal function. He has erred in theory, perhaps, by being too schematic, and in practice by relying on unreliable methods. Thus Rowntree and Fitz[5] state that the hypobromite method for urea in the blood, as employed by Widal, is subject to an error of from 10 to 60 per cent. Folin,[6] within the last year, by introducing chemical methods which permit of rapid and accurate analyses of both urea and total non-protein nitrogen in minimal quantities of blood, has greatly extended the possibilities of this mode of examination.

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[1] Bright, R., *Guy's Hosp. Rep.*, 1836, i, 338.

[2] Müller, F., *Verhandl. d. deutsch. path. Gesellsch.*, 1905, ix, 64.

[3] Strauss, H., Die chronischen Nierenentzündungen, Berlin, 1902.

[4] Widal, F., *Bull. et mém. Soc. méd. d. hôp. de Paris*, 1911, xxxii, series 3, 627.

[5] Rowntree, L. G., and Fitz, R., *Arch. Int. Med.*, 1913, xi, 258.

[6] Folin, O., and Denis, W., *Jour. Biol. Chem.*, 1911–12, xi, 527.

The present report deals with the results of our study of the blood by Folin's methods in a series of fifty-nine hospital patients.[7] Our main concern has been with nephritis, but we have examined the blood in many other conditions, as opportunity offered.

Throughout the paper we use the term non-protein nitrogen to indicate the nitrogen not precipitable by the methyl alcohol and zinc chloride of Folin's method. The terms retention nitrogen, waste nitrogen, or extractive nitrogen should perhaps be discarded in favor of descriptive appellations, such as non-protein nitrogen, incoagulable nitrogen, or simply filtrate nitrogen. This has been suggested by Folin's experiments which indicate that a small fraction of the non-protein nitrogen may be not a waste product, but amino acids available for utilization by the body cells. The unmodified term urea is used to signify the ammonia-urea fraction; that is, the nitrogen determined by the urea method employed. Separate estimations of the ammonia were not made as they seem not to be of clinical value. According to Folin, the older methods exaggerated the quantitative importance of the ammonia fraction. We used the technique described by Folin and his collaborators, and we met with no difficulties of importance.

METHOD.

The principal steps in the analyses will be briefly enumerated, but the minor details, which, however, are essential in practice, will be omitted. The total nitrogen method is a miniature Kjeldahl method, while the urea method is an analogous modification of standard procedures. Accuracy is secured by careful measurements (special pipettes) and by substituting Nesslerization for titration in the final step. Minute amounts of ammonia which it would be impossible to estimate by the usual titrations are thus accurately determined.

A. Collection of Blood.—(1) 10 c.c. of blood are added to about 60 c.c. of pure methyl alcohol in a 100 c.c. volumetric flask, and after being shaken are diluted to volume with the same reagent. (2) After a few hours the coagulated blood is filtered off and the filtrate clarified by the addition of a few drops of zinc chloride solution, and is filtered again. Each 10 c.c. of filtrate represents 1 c.c. of the original blood.

B. Total Nitrogen Determination.—(1) 10 c.c. of the filtrate are measured into a large Jena test-tube and freed from alcohol by immersion in boiling water. (2) The organic matter is destroyed and the nitrogen converted into ammonium

[7] University of Pennsylvania Hospital, service of Dr. Alfred Stengel, and Philadelphia General Hospital, service of Dr. David Riesman.

sulphate by digesting with potassium sulphate and concentrated sulphuric acid over a microburner. The fumes are carried off by special absorption tubes connected with a suction pump. (3) The ammonia is set free by the addition of an excess of sodium hydrate solution and is driven over into dilute hydrochloric acid by a powerful air blast. (4) The acid solution is transferred to volumetric flasks of suitable volume, treated with a definite quantity of dilute Nessler's solution, and made up to volume. (5) The deep amber color that develops is compared, by the aid of a colorimeter, with that of an ammonium sulphate solution of known strength, similarly treated. From the colorimetric readings the amount of nitrogen is easily calculated.

C. Urea Determination.—The urea method is similar in most respects. The alcohol is removed by the aid of moderate heat (50° C.) and an air current. A higher temperature is avoided as it might decompose the urea. Digestion is then carried on by the aid of acetic acid and fused sodium acetate at a temperature of 150° C. A condenser is used and the proper heat regulated by the aid of a temperature indicator which is sealed in a glass ampoule and floated on the boiling fluid. The remaining steps are almost identical with those used for the total nitrogen. As we have previously stated, the figures for urea thus obtained include the preformed ammonia.

The results in both cases are stated in terms of milligrams per 100 c.c. of blood. In the literature these values are occasionally stated as grams per liter. Where we have had occasion to refer to such figures we have converted them into milligrams per 100 c.c.; *i. e.*, 0.25 gm. per liter is equal to 25 mg. per 100 c.c.

In collecting the blood from the vein we used an accurately graduated syringe, and in most instances we removed 10 c.c. (diluted to 100 c.c.) rather than the smaller quantity originally recommended. This has supplied sufficient filtrate to permit of duplicates both for the nitrogen and urea estimations and also for retests when required. However, 2 c.c. would suffice for the nitrogen estimation in case of necessity.

In order to permit of many examinations at one time we installed an apparatus providing space for eight simultaneous digestions of either nitrogen or urea. A rocking bar containing the tubes leading to the glass suction pump allowed adjustment of the fume extraction tubes without danger of breaking, a convenient modification of the original apparatus. As we also provided an equal number of evaporating and blow over units, it was possible for two persons to complete from twenty-four to thirty-two tests in an afternoon, or, in other words, to examine six or eight specimens for both urea and total filtrate nitrogen with duplicates in each case. The evaporation of the alcohol from the specimens consumes the most time, particularly in the case of urea. This can be lessened for the urea if the capillary tubes are made of fairly large caliber. It is very important to have good test-tubes of thin Jena glass (Schott); thick tubes, even of the best quality, are of no use and at first caused much delay and the loss of several important specimens. We used large round bulbs[8] on our blow over tubes instead of the vertical pipette-like ones originally recommended. As a result, we never had the slightest loss of fluid with the strongest air current. Also we have found it essential to cover the rubber stopper con-

[8] This was suggested by Mr. Farmer, of Dr. Folin's laboratory.

nections with water before the air is turned on so as to detect at once any loss by leakage. In Nesslerization we used the Winkler modification and we had no difficulty with cloudiness except rarely after too rapid mixing or in too concentrated solutions.

Control experiments were made on a watery solution of urea which theoretically should have shown 50 mg. of nitrogen per 100 c.c., and which gave the following results by the total nitrogen method: 49.7, 49.5, 50.0, and 49.7 mg. With the urea method results were far less uniform and here slight variations in technique were found to be of great moment. Eventually satisfactory analyses by the urea method gave 50.0 and 47.4 mg. Under conditions analogous to those used in blood examinations, *i. e.*, in methyl alcohol solution, the results by the total nitrogen method were 47.7 and 49.8 mg. In actual blood examinations we found the greatest range of error when it was necessary to Nesslerize in small volumetric flasks (25 or 10 c.c.). This we attributed to the incomplete washing of delivery tubes and receivers necessitated by the narrow margin of fluid available. Taking our duplicate results serially in forty-four consecutive examinations, we found that the total nitrogen estimations showed variations from the average ranging up to 2 mg. per 100 c.c. of blood, and the urea up to 1.5 mg. The average difference between duplicates in total nitrogen estimations was 1.6 mg. per 100 c.c. of blood, and in the urea estimations 1.4 mg.

Analyses of the various reagents by means of blanks showed appreciable impurities in the potassium sulphate and kerosene; in the quantities used they introduce an error which when calculated for 100 c.c. of blood amounts to about 2 mg.; the quantity of these reagents used is the same in every analysis. Finally we have convinced ourselves by experiment that the slight brownish cloud, which occasionally forms in the filtrate on long standing, does not represent an appreciable quantity of nitrogen.

Before reporting our own figures we shall present some results obtained with the older methods, as used by other investigators. The principal variation in the methods employed has been in the manner of precipitating the albumin. Serum has generally been preferred to blood, and the nitrogen estimations have almost invariably been made by the Kjeldahl method, variously modified. The methods of precipitation are of several types. Widal's method, as used by Rowntree and Fitz,[9] was as follows: 10 c.c. of blood were added to 115 c.c. of 95 per cent. alcohol, 100 c.c. of the filtrate representing 8 c.c. of blood. This was evaporated to dryness preparatory to making the estimation by the Kjeldahl method. Jackson and Saiki[10] used a similar technique, but washed and ground up the coagulated blood protein with 95 per cent. alcohol, after which filtrate and washings were united. Limbeck and Pick,[11] who seem to have been the first to use the Kjeldahl method for estimating the nitrogen of the blood, used an alcohol extraction method. Strauss[12] ran the blood serum into five volumes of boiling distilled water and then added an amount of 2 per cent. acetic acid previously determined to be sufficient to cause a flaky coagulation. The solution

[9] Rowntree, L. G., and Fitz, R., *loc. cit.*

[10] Jackson, H. C., and Saiki, T., *Arch. Int. Med.*, 1912, ix, 79.

[11] Limbeck and Pick, cited by Ascoli, G., Vorlesungen über Urämie, Jena, 1903.

[12] Strauss, H., *loc. cit.*

TABLE I.

Author.	Diagnosis.	No. of cases.	Mg. of total nitrogen per 100 c.c.		Notes.
			Maximum.	Minimum.	
Strauss	Chronic interstitial nephritis	28	116	82	
Strauss	Chronic interstitial nephritis with uremia	19	266	68	
Strauss	Chronic parenchymatous nephritis	10	152	25	
Strauss	Chronic parenchymatous nephritis with uremia	4	80	36	
Strauss	Transitional forms	5	75	38	
Strauss	Transitional forms with uremia	14	79	25	
Strauss	Liver diseases	4	112[13]	29	
Strauss	Uncompensated heart disease	8	70	31	
Strauss	Febrile diseases	6	91	48	
Strauss	Miscellaneous diseases	6	76	22	
Strauss	Normal individuals	5	34	23	
Ascoli	Non-nephritics	24	184	19	This includes many cases from the literature, but not Strauss's.
Ascoli	Nephritics	30	212	14	
Obermayer and Popper	Uremia	30	658	33	Only five below 100.
Obermayer and Popper	Nephritis without uremia	5	350	56	
Obermayer and Popper	Normal individuals	2	36	35	
Obermayer and Popper	Pneumonia	8	297	39	
Obermayer and Popper	Septic diseases	4	119	44	
Obermayer and Popper	Heart failure and emphysema	7	290	24	
Obermayer and Popper	Cerebral disease	10	81	33	
Obermayer and Popper	Liver diseases	4	77	42	
Hohlweg	Healthy kidneys	10	60	41	
Hohlweg	Nephritics	12	93	50	
Hohlweg	Uremia (with recovery)	4	96	62	
Hohlweg	Uremia (fatal)	9	340	117	
Hohlweg	Fatal nephritis without uremia	7	370	144	
Hohlweg	Moribund cases not dying of nephritis	11	95	49	
Rowntree and Fitz	Nephritics	5	280	30	
Rowntree and Fitz	Passive congestion with and without nephritis	8	280	60	
Rowntree and Fitz	Normal individuals	3	70	30	
Monakow		2	98	83	
Foster	Nephritics	16	393	43	
Foster	Normal kidneys	5	76	33	
Widal	Nephritics	31	—	200	Urea only was determined (hypobromite method).

[13] Agonal.

was then filtered, washed with distilled water, tested for albumin, and evaporated to a fixed volume. Folin has recently simplified this process in connection with his uric acid method,[14] but he does not believe that the filtrate is uniformly free from albumin. Obermayer and Popper[15] acidified 20 c.c. of blood with dilute acetic acid, and boiled, filtered, and washed the solution with hot water. The filtrate and wash water were united. Hohlweg[16] added a special reagent consisting of acetic acid and monocalcium phosphate, and boiled the solution after half saturation with sodium chloride.

In table I are given a few figures collected from the literature, indicating the range of non-protein nitrogen found by these methods in the blood in different conditions.

It will be noticed that almost all the results reported range much higher than our own. We thought that this might be due to the use of serum instead of whole blood, as in our work. To determine this point we made parallel observations on two cases taking both whole blood and serum and analyzing each by Folin's method, with the following results:

No. of case.	Whole blood.	Serum.
69	34.8	41.3
75	30.0	30.0

These results, therefore, do not explain the higher results obtained by those who have worked with serum. Folin attributes the discrepancy to the faulty methods of precipitation generally employed. The figures that follow (table II) are from Folin's recent article,[17] and, as they are based on the same method, are therefore more strictly comparable with our own.

TABLE II.

Diagnosis.	No. of cases.	Mg. of total nitrogen per 100 c.c.		Mg. of urea nitrogen per 100 c.c.	
		Maximum.	Minimum.	Maximum.	Minimum.
Normal individuals	16	26	22	13	11
Syphilitics	63	45	22	26	13
Insane	21	62	24	46	13
Nephritics	11	96	38	68	19

[14] Folin, O., and Denis, W., *Jour. Biol. Chem.*, 1912–13, xiii, 469.
[15] Obermayer, F., and Popper, H., *Ztschr. f. klin. Med.*, 1911, lxxii, 332.
[16] Hohlweg, H., *Deutsch. Arch. f. klin. Med.*, 1911, civ, 216.
[17] Folin, O., and Denis, W., *Jour. Biol. Chem.*, 1913, xiv, 29.

Folin states that renal lesions were not by any means excluded in all cases. The urea, on the average, constituted from 50 to 56 per cent. of the total non-protein nitrogen; in the most marked cases of nitrogen retention it went up to 70 per cent.

In table III are summarized the essential features of the fifty-nine individuals that we have studied. They fall readily into four divisions: (1) those showing no disturbance of renal function; (2) those with marked cardiovascular disease of some type, a certain number of which show urinary changes as the result of renal congestion; (3) those showing nephritis; and (4) those in which certain features lead one to suspect nephritis, but in which the existence of nephritis is not certain.

All the cases of group 1 were free from definite evidences of renal disturbance. Four showed an occasional trace of albumin in the urine (table III). Examination of the non-protein nitrogen column (table III) shows that all the figures lie between 15 and 43. The ammonia-urea fraction, if we except one case of acute leukemia, constitutes less than 70 per cent. of the total non-protein nitrogen.

Group 2 (table III) falls naturally into three subdivisions: (*a*) individuals with cardiac disease, either myocardial, valvular, or pericardial; (*b*) those with aneurysm (2); (*c*) those in which arteriosclerosis was the most conspicuous feature, associated, however, with myocardial weakness. Most of the individuals of group 2 showed albuminuria and casts, as a rule small in quantity. Of the four cases in which phthalein elimination was estimated two showed a moderately reduced output. The blood pressure was within normal limits or low (divisions *a* and *b*); above normal (division *c*). Examination both of the non-protein nitrogen and of the ammonia-urea percentage shows that the figures lie within the same range as those of group 1. The series is too small for final conclusions, but it suggests that nitrogen retention does not, as a rule, accompany disturbances of renal function associated with chronic passive congestion, and that we may have here a method of distinguishing more serious renal involvement from mere congestion.

TABLE III.

Group 1.

Serial No. of case.	Diagnosis.	Age in yrs.	Sex.	Total nitrogen in mg. per 100 c.c.	Urea nitrogen in mg. per 100 c.c.	Urea per cent.	Phthalein per cent., in 2 hrs.	Blood pressure.	Eyegrounds.	Albumin.	Casts.	Specific gravity.	Hemoglobin per cent.	Red blood cells.
20	Post-operative adhesions	18	F.	15.9	7.7	48.4								
19	Chronic gastritis	34	F.	20.4	10.6	52.0		122–90		0	0	1,024	85	5,200,000
33	Arthritis deformans	32	F.	21.0	11.9	56.7							70	4,000,000
43	Mediastinal gumma	44	M.	21.7	12.3[18]	56.7		132–85		+	0		79	4,400,000
60	Gonorrheal arthritis	27	M.	25.2	12.3	48.8		115–65		0	0	1,025	90	5,610,000
37	Chronic tonsillitis	14	M.	25.3	11.2	44.3		140–70		0	0		87	5,400,000
51	Arthritis deformans	42	F.	26.8	14.6	54.5								
2	Gout	49	M.	27.2[18]	18.0[18]	66.2				0	0	1,026	90	4,100,000
54	Malarial cachexia	41	M.	27.2	13.4	49.3		90–50		0	0		61	3,950,000
6	Tabes dorsalis	50	M.	27.5[18]	16.6[18]	60.4			Optic atrophy	0	0	1,019		
18	Acute lymphatic leukemia	34	M.	29.1	21.1	72.5		115–55		+	0	1,013	25	1,570,000
7	Tabes dorsalis	58	M.	29.2[18]	15.5[18]	53.1			Normal	0	0	1,020	85	3,400,000
32	Chronic colitis	37	M.	29.4[18]	—			116–65		0	0	1,025	86	5,700,000
8	Gout	46	M.	32.9[18]	—					0	0	1,012	90	5,200,000
77	Sciatica	29	M.	38.9	23	59.1		120–60		0	0	1,040	73	5,250,000
3	Tuberculous meningitis	25	M.	40.0[18]	—			135–		+	0	1,029		5,350,000
80	Hypertrophic arthritis (knee)	52	M.	42.4	23.5[18]	55.6		122–70		+	0	1,021	80	4,900,000

Group 2.

a.

42	Post-influenzal myocarditis, generalized edema, hydrothorax	25	M.	23.4	15.0	64.1	52	120–85		++	+	1,027	87	5,500,000
47	Adhesive pericarditis, general anasarca	46	M.	26.0[18]	14.3[18]	55.0		105–60		+	+	1,027	84	5,100,000
79	Aortic insufficiency	51	M.	26.0	15.8[18]	60.8	30 / 35 } 65	165–60		++	+	1,031	58	4,050,000
58	Aortic insufficiency (lues)	30	M.	31.3	—			140–68		+	+	1,015	73	3,910,000
9	Aortic insufficiency	47	M.	33.7	22.2[18]	65.9		135–		0	0	1,021	95	4,300,000

[18] No duplicate estimations.

TABLE III.—*Continued.*

Group 2.

b.

Serial No. of case.	Diagnosis.	Age in yrs.	Sex.	Total nitrogen in mg. per 100 c.c.	Urea nitrogen in mg. per 100 c.c.	Urea per cent.	Phthalein per cent., in 2 hrs.	Blood pressure.	Eyegrounds.	Albumin.	Casts.	Specific gravity.	Hemoglobin per cent.	Red blood cells.
1	Aneurysm of aorta	54	M.	26.0[19]	—			138–112		0	0	1,018		4,300,000
53	Aneurysm of aorta	54	M.	30.6	—			135–60		+	+		66	3,950,000

c.

75	Arteriosclerosis, auricular fibrillation	50	M.	30.0	15.6	52.0	8 / 30 } 38	150–90		++	++	1,011	70	5,350,000
										+	0	1,023		
5	Hemiplegia, sarcomatosis	63	M.	31.0[19]	17.5[19]	56.5								
40	Arteriosclerosis, myocardial insufficiency	67	M.	39.2	24.4	62.2	35	165–100	Early cataract / Retinal sclerosis	+	+	1,017	82	4,200,000
25	Arteriosclerosis, myocardial insufficiency, edema	53	M.	25.1	—			190–130	Retinal sclerosis	+	+	1,019		

Group 3.

a.

35	Mitral stenosis, chronic parenchymatous nephritis	18	M.	29.9	15.3[19]	51.2	35 / 28 } 63	138–100		+++	+++	1,013	75	5,000,000
14	Congenital pulmonary stenosis, chronic parenchymatous nephritis	20	M.	40.3	29.5	72.2	36	135–75	Normal	+++	++	1,015	60	3,300,000

b.

4	Chronic nephritis, chronic uremia, cardiac insufficiency	50	M.	40.0[19]	15.0[19]	37.5		200–	Retinal sclerosis / Old hemorrhage	++	+	1,023		5,350,000
68	Chronic nephritis, arteriosclerosis, cardiac insufficiency	59	M.	40.5			30 / 15 } 45	225–120		++	+++		75	4,900,000

[19] No duplicate estimations.

Group 3.

b.—Continued.

Serial No. of case.	Diagnosis.	Age in yrs.	Sex.	Total nitrogen in mg. per 100 c.c.	Urea nitrogen in mg. per 100 c.c.	Urea per cent.	Phthalein per cent., in 2 hrs.	Blood pressure.	Eyegrounds.	Albumin.	Casts.	Specific gravity.	Hemoglobin per cent.	Red blood cells.
13	Chronic nephritis, uremic convulsions, podagra	52	M.	41.6	28.9	69.5		225–	Retinal sclerosis; Early optic atrophy	+	+	1,010		
12	Chronic nephritis, arteriosclerosis	56	M.	44.3	26.1	58.6		215–120	Retinal sclerosis	++	++	1,009	98	6,700,000
11	Chronic nephritis, cardiac insufficiency	59	M.	44.9	34.4	76.6		190–110	Normal	+++	++	1,020	75	4,100,000
62	Chronic nephritis, arteriosclerosis, hemiplegia	54	M.	53.4	33.3	61.8	29, 9, 34	215–120		+	++	1,019	64	3,780,000
24	Chronic nephritis, chronic uremia	50	M.	54.7	35.0	64.0		185–88		+	+	1,010		
52	Chronic nephritis, retroperitoneal sarcoma	54	M.	66.2[20]	42.4[20]	64.1	20, 14, 34	148–85	Retinal sclerosis	++	++	1,025	69	3,800,000
48	Chronic nephritis	61	F.	71.0	56.8	80.0	10	240–190	Neuroretinitis; Hemorrhages	++	++	1,009	54	4,700,000
39	Chronic nephritis, arteriosclerosis	54	M.	82.3	59.9	72.8	2	215–110	Albuminuric retinitis	+	++	1,013	75	4,500,000
34	Chronic nephritis, arteriosclerosis	51	M.	103.5	82.1	79.3	1	145–85	Normal	0	0	1,009	61	3,800,000
76	Chronic nephritis, gout	48	M.	120.9[20]	93.9[20]	72.8	0, 1, 1	210–105		++	(Pus)	1,012	32	2,500,000

c.

17	Chronic nephritis	35	M.	48.3	34.7	71.9		102–60		+	+	1,017		3,100,000
23	Chronic nephritis, convulsions, pulmonary tuberculosis	36	M.	48.4	34.2	70.7		195–		++	+	1,016		
10	Chronic nephritis	29	M.	50.0	34.3	68.6		102–55		++	+	1,016		
44	Chronic nephritis	35	M.	70.1[20]	63.0[20]	90.0	2	194–115	Normal	++	++		57	3,400,000

[20] No duplicate estimations.

TABLE III.—*Continued.*

Group 3.

c.—Continued.

Serial No. of case.	Diagnosis.	Age in yrs.	Sex.	Total nitrogen in mg. per 100 c.c.	Urea nitrogen in mg. per 100 c.c.	Urea per cent.	Phthalein per cent., in 2 hrs.	Blood pressure.	Eyegrounds.	Albumin.	Casts.	Specific gravity.	Hemoglobin per cent.	Red blood cells.
41	Chronic nephritis	32	M.	72.3	61.4	85.5	Trace	210–135	Albuminuric retinitis	+	+	1,010	33	2,600,000
49	Chronic nephritis, cardiac insufficiency	36	F.	160.0[21]	136.0	85.1	Trace	190–120	Normal	+	0	1,010	41	2,600,000
31	Chronic nephritis, uremia, delirium tremens	30	M.	181.0	144.0	79.9		95–58	Disc hazy, veins distended, retinal hemorrhages	+	+	1,017	75	3,800,000
							d.							
*21	Acute nephritis	15	M.	149.0	105.0[21]	70.4		132–95		++	+++	1,021	74	3,700,000
							e.							
63	Amyloid disease	23	M.	99.0			Trace	94–62		++	++	1,013	49	3,580,000
							Group 4.							
66	Syphilis, tertiary, anemia	38	M.	20.1	12.4	61.7		100–60		+	+	1,011	44	2,180,000
78	Acute cholecystitis	29	M.	23.7	10.0[21]	42.2	8.5 / 22.5 } 31	165–95		++	+		62	4,550,000
67	Atrophic cirrhosis	55	M.	23.8	6.9	29.0		126–82		+	+	1,020	68	3,820,000
16	Coma (uremic?), death	48	M.	27.9	13.5	48.4		165–105		++	+			
56	Hypertrophic cirrhosis, delirium tremens	36	M.	30.3	15.3[21]	50.5	15 / 5 } 20	145–70	Pallor of disc, distended veins, hemorrhage	+	+	1,028	55	3,500,000
57	Acute bronchitis, transposition of viscera	29	M.	30.5	19.4[21]	63.6		125–80		+	+	1,013	81	4,730,000
36	Early nephritis	48	M.	33.5	17.2	51.3	65	160–80	Normal	+	+	1,013	83	5,000,000
61	Idiopathic epistaxis, secondary anemia	24	M.	35.2	20.0	56.8		120–60		+	+	1,023	64	3,780,000

[21] No duplicate estimations.

Group 3 falls into five divisions: (*a*) chronic nephritis with marked albuminuria and cylindruria, marked edema, and little if any increase in blood pressure; (*b*) chronic nephritis associated with marked arteriosclerosis and elevated blood pressure,—all cases over fifty years of age; (*c*) chronic nephritis in younger individuals, not conspicuously sclerotic, with high blood pressure as a rule, with anemia, and with the frequent appearance of a few red blood cells in the urine; (*d*) acute nephritis; (*e*) amyloid kidney. In both cases of the first subgroup, *a*, cardiac complications existed which render the findings somewhat difficult of interpretation; possibly they should be classified in group 2. In both individuals the non-protein nitrogen and the ammonia-urea percentage lay within the limits established for groups 1 and 2. Case 35 is of interest because of the constant presence in the urine of enormous numbers of epithelial and fatty casts and even of free fat droplets. Both cases averaged about seven grams of albumin per liter by Esbach's method. All the cases of subgroup 3, *b*, except one, showed albumin and casts in the urine, although at times only scantily. The specific gravity was low as a rule. In the majority, retinal changes existed. Anemia was not a conspicuous feature of the group except in two cases. The blood pressure was above normal. The phthalein elimination, in the few cases in which it was determined, was low. Inspection of the non-protein nitrogen figures shows them to be 40 or above; the ammonia-urea percentages, except in case 4, lie either at the extreme upper level of the range found in group 1, or distinctly above it. In this group, therefore, we find for the first time constant evidence of nitrogen retention. It is of interest to note that the case showing next to the highest figures for non-protein nitrogen and the highest ammonia-urea percentages, as well as an extremely low phthalein elimination, showed no elevation of blood pressure, no eyeground changes, and no evidences of nephritis in the urine. The further course of this individual is being followed with special interest.

The cases of subgroup 3, *c*, all belong to that type of nephritis in which pallor is a characteristic feature and which usually shows also puffiness beneath the eyes. The urine showed more or less albumin and, with the possible exception of case 49, casts. In the two cases

not showing blood pressures well above normal, there was definite cardiac decompensation. With improvement in the general condition a marked rise of blood pressure occurred. The three cases on which phthalein elimination was estimated showed an extremely low output. All the cases showed a non-protein nitrogen above 40 and an ammonia-urea percentage above 70. That there is a fixed relation between the degree of nitrogen retention and the prognosis can not be claimed, since case 31 with the highest degree of retention, under active treatment, improved rapidly. The uremic condition and the delirium tremens cleared up and later an examination of the blood showed a non-protein nitrogen of only 36. Subgroup 3, *d*, containing a single case, shows marked nitrogen retention.

Group 4 contains eight cases in which nephritis may be suspected. In all the urine showed albumin and casts. In case 56 the findings, as well as the low phthalein elimination, were possibly due to cardiac decompensation which complicated the condition at the time the observations were made. In case 16 the diagnosis of uremia was based entirely on the data presented in the table. The patient died within an hour or two after admission, and no clinical history was obtained; an autopsy was not permitted. In the other cases some of the findings suggested a very early stage of nephritis. In none of the eight cases is there evidence of nitrogen retention.

From the foregoing results it appears that there is a definite tendency toward nitrogen retention in most cases of nephritis. This is apparently least marked in the so called chronic parenchymatous nephritis in which the disturbance is one of fluid retention rather than of poor urea elimination. In the cases in which nitrogen retention is evident the higher percentage of the ammonia-urea fraction indicates that it is principally the excretion of this fraction that is impaired. The rapid fluctuations that we have observed in two cases from which blood was repeatedly taken indicate that rapid variations in the concentration of the non-protein nitrogen of the blood may occur.

Mere passive congestion of the kidneys produces apparently albuminuria and even casts and causes some impairment in the elimination of phenolsulphonephthalein without producing a retention of non-protein nitrogen.

We have in this method an additional aid, and, we believe, a valuable one for ascertaining the state of activity of the kidneys. On the other hand, we are not warranted in inferring any constant relation between the existence or imminence of uremia and the degree of nitrogen retention. The existence of uremia without an increase in the non-protein nitrogen is, we believe, unlikely.

CONCLUSIONS.

1. In a series of non-nephritic individuals the total non-protein nitrogen of the blood, determined by Folin's method, was found to lie between 15 and 43 milligrams per 100 cubic centimeters. From 50 to 60 per cent. of this was in the ammonia-urea fraction.

2. In cardiovascular disease with renal congestion, but without other renal lesion, there was no evidence of increase of non-protein nitrogen in the blood, nor of alteration of the ammonia-urea percentage.

3. In chronic nephritis with marked albuminuria and edema there was very little, if any, increase or alteration.

4. In chronic nephritis with hypertension the non-protein nitrogen was definitely increased, ranging from 40 to 180 milligrams per 100 cubic centimeters of blood. The percentage of the ammonia-urea fraction was usually higher than in non-nephritic cases.

5. Cases showing high non-protein nitrogen values were subject to rapid fluctuations in these values in the course of a few days. As a rule, clinical improvement was associated with a fall of the non-protein nitrogen figures to nearer the normal range.

6. Uremia was almost always accompanied by an increase of non-protein nitrogen in the blood, but no constant relation could be established between the degree of increase and the tendency to uremia.

7. Our cases have not yet been followed for a long enough period to admit of conclusions as to the possible relation between the degree of non-protein nitrogen retention and ultimate prognosis.

8. We believe this method to be a valuable aid in the clinical study of nephritis and that it can be readily carried out in any well equipped clinical laboratory.

A STUDY OF THE CEREBROSPINAL FLUID IN ACUTE POLIOMYELITIS.*

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In a paper published last year Draper and Peabody[1] reported on the examination of the cerebrospinal fluid in sixty-nine cases of acute epidemic poliomyelitis in the Hospital of The Rockefeller Institute for Medical Research. The present study deals with their findings and, in addition, with the results of the examination of fifty-seven more cases that were admitted to the hospital during the summer of 1912. The purpose of this study was to ascertain whether the examination of the spinal fluid may be of aid in early diagnosis and whether the abnormalities present have any relation to the severity of the disease, the localization of paralysis, and the prognosis.

In all, 362 examinations have been made in 126 cases. The points to which attention was paid were the macroscopic appearance of the fluid, the number and type of cells present, the presence of globulin, and the power of reducing Fehling's solution. In all cases lumbar puncture was performed as soon after admission as possible, and at varying intervals afterwards, in some cases on successive days. The period covered extends from the preparalytic stage to the twelfth week after onset. The amount of fluid withdrawn varied from a few cubic centimeters to thirty, and although no special precautions were taken in limiting the amount withdrawn, no inconvenience was suffered by the patients, who, as a rule, slept quietly for a few hours after being returned to bed. Manometric estimation of the pressure of the fluid was found to be unsatisfactory, as the patients were usually crying, and this rendered observations as to the rapidity of flow also unsatisfactory; but as far as

* Received for publication, May 14, 1913.

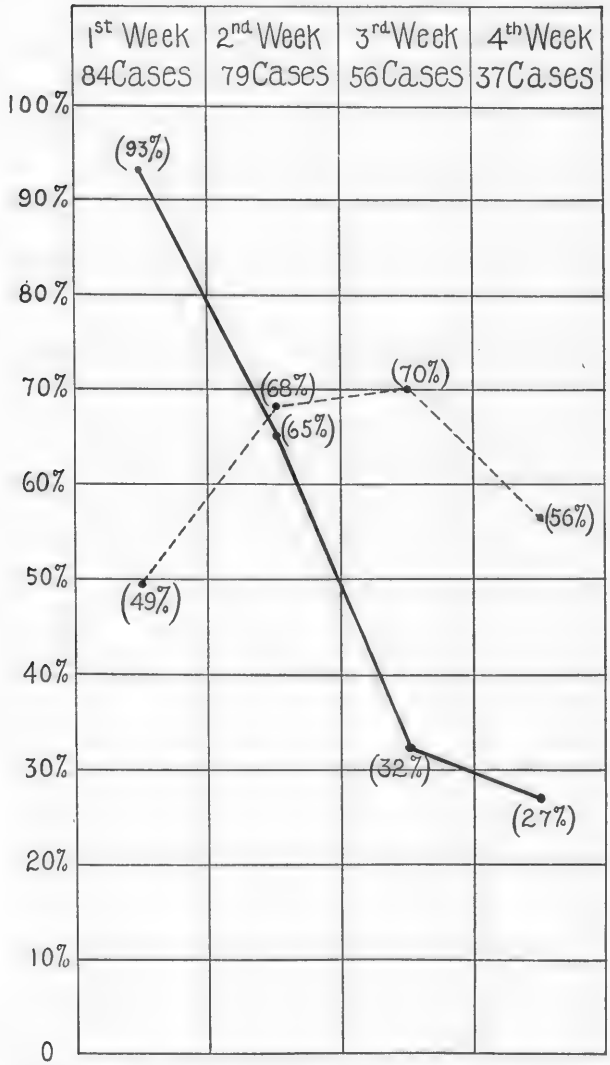
[1] Draper, G., and Peabody, F. W., *Am. Jour. Dis. Child.*, 1912, iii, 153.

could be judged from the rate of flow in the quieter or very ill patients, an especially high pressure was not usually present. In these patients a continuous stream lasted usually only a few seconds, following which the flow from the needle was in a steady stream of drops. The fluid was always examined immediately after withdrawal.

The following method of estimating the number of cells was employed: The fluid was mixed with acetic acid in a hematocytometer pipette for white blood corpuscles. 50 per cent. acetic acid was drawn up to the "1" mark on the stem and the pipette was then filled with the spinal fluid. After a thorough shaking two counting chambers were filled immediately and counts were made. The globulin was estimated by the butyric acid method of Noguchi, and the intensity of the reaction was noted as very slightly plus (v. s. +), denoting a faint cloud; slightly plus (s. +), a definite cloud settling to a fine precipitate; plus (+), a precipitate forming immediately; and double plus (+ +), a heavy flocculent precipitate. The reaction with Fehling's solution was tested by boiling one cubic centimeter of spinal fluid with one cubic centimeter of Fehling's solution and allowing the mixture to stand for ten or fifteen minutes. No quantitative estimation of the reducing power was attempted. Reduction took place with every fluid examined.

The macroscopic appearance of the fluids was constant; nearly all were clear and colorless, a few showed very slight opalescence, and a few contained small white flakes. Those containing the small white flakes were usually obtained in the later stages of the disease. On standing, a delicate clot formed in a very small number of cases, but no relation between this property and the globulin reaction, cell count, or severity of the disease could be made out.

Owing to the lack of precise knowledge of the character of the spinal fluid in normal children and in children suffering from other acute infections, it was necessary to adopt a somewhat arbitrary standard as to the changes to be regarded as pathological. A globulin reaction noted as slightly plus (s. +) or more has been considered abnormal, while all those noted very slightly plus (v. s. +) and negative (—) have been considered normal. Cell counts of over ten cells to the cubic millimeter have been considered as ab-



TEXT-FIG. 1. Curves showing variation in cell count and in globulin content in the cerebrospinal fluid in acute poliomyelitis. ——— = per cent. of cases with cell count above normal; - - - - = per cent. of cases with globulin content above normal.

normal, and those of ten or less as normal. With these standards it is improbable that a normal fluid has been classed as abnormal.

In text-figure 1 are shown two curves; the continuous line indicates the percentage of cases that showed abnormal cell counts in each week of the disease, while the interrupted line indicates the percentage of cases that showed abnormal globulin reactions in each week. The percentage was calculated from the number of cases examined in each week, but if the fluid from a given case was examined more than once during a week, only the highest figure was taken. From the curves it is seen that while the number of cells was highest early in the disease and gradually diminished later, the globulin reaction was at a maximum in the third week of the disease and then gradually diminished. Of the fluids from the eighty-four cases examined in the first week, seventy-eight, or 93 per cent., showed cell counts of over ten per cubic millimeter, and forty-one, or 49 per cent., showed a globulin reaction of slightly plus (s. +) or over; of the fluids from the seventy-nine cases examined in the second week fifty-one, or 65 per cent., showed abnormal cell counts, and fifty-four, or 68 per cent., showed abnormal globulin reactions; of the fluids from the fifty-six cases in the third week eighteen, or 32 per cent., gave abnormal cell counts, and thirty-nine, or 70 per cent., abnormal globulin reactions; of the fluids from the thirty-seven cases in the fourth week ten, or 27 per cent., gave abnormal cell counts, and twenty, or 54 per cent., abnormal globulin reactions. After the fourth week examinations were made only on cases showing persistent abnormalities, so that the figures for the later weeks have not been included; twenty-five cases were examined in the fifth week and twenty-four in the sixth week or later.

Of the eighty-four cases seen in the first week, in fifty the number of cells in the spinal fluid was fifty or more per cubic millimeter, while in six the number of cells was not increased. In one of the cases, seen on the seventh day of the disease and the third of paralysis, the spinal fluid contained but seven cells per cubic millimeter and gave a + globulin reaction. There was respiratory involvement and the patient died a few hours after the examination was made. Another one of the cases was classed among the abortive cases and the spinal fluid was never abnormal. In another the cell count was

never abnormal, but a + + globulin reaction was present. In three other cases the spinal fluid later became abnormal. In one abortive case, seen on the second day of the disease, the number of cells in the spinal fluid was not increased, but on the following day the fluid contained sixty-two cells per cubic millimeter. Seventy-nine fluids from patients in the second week of the disease were examined, and in fourteen, or 18 per cent., the cells numbered fifty or more per cubic millimeter. Counts as high as this were found only once or twice among the fluids that were obtained in the later weeks. Of the fluids obtained during the third week, only 32 per cent. showed abnormal cell counts, while 70 per cent. showed abnormal globulin reactions. The most usual type of fluid examined was one with a moderate increase in the number of cells and a definite increase in the amount of globulin, but many fluids, especially in the earlier stages, showed a great increase of cells and only a slight increase of globulin that would be noted as s. + or v. s.+. Another quite distinct type is that of fluids containing less than ten cells per cubic millimeter and giving a globulin reaction of + or + +. The highest cell count noted was one of 1,221 per cubic millimeter on the second day of the paralysis; three other fluids contained over 500 cells per cubic millimeter, and twenty-seven contained between 500 and 100. Of these thirty-one fluids only twelve, or 39 per cent., gave a globulin reaction of + or over. Sixteen cases at no time showed an abnormal cell count and of these nine, or 56 per cent., showed a globulin estimation of + or + +, while in one fluid it was noted as + + +. The globulin estimation has been of great value in cases seen for the first time late in the disease or in cases where the number of cells was never very high.

The increase in the number of cells was due in nearly every case to mononuclear cells. A comparison of the results obtained by examining the cells in the hematocytometer chamber and in a stained dry preparation showed that while the chamber method gave an approximate idea of the proportion of polymorphonuclear cells present, it was quite inefficient for differentiating the various kinds of mononuclear cells. The dry preparations were made by centrifuging a mixture of one part of spinal fluid and two parts of carefully centrifuged sterile serum, pouring off the supernatant fluid,

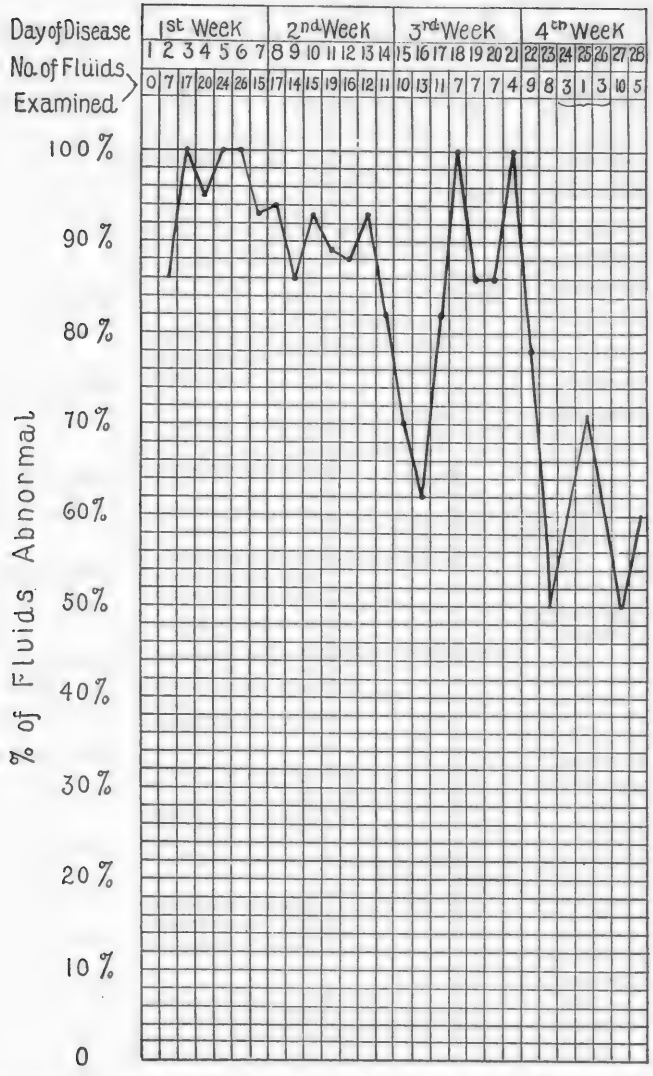
collecting the deposit in a fine capillary glass tube, and spreading it between two cover-glasses as in preparing a blood film. The staining method employed was similar to the May-Giemsa method of Pappenheim for blood films. The cover-glass preparations were fixed in Wright's or Hasting's solution for three minutes, and stained by the addition of an equal quantity of water for one minute. The fluid was then poured off and without washing the film was stained in Giemsa solution (1 to 15 dilution) for ten to fifteen minutes, the last stages of the process being watched under the low power of the microscope. By this method the cells were obtained well spread and in an apparently undamaged condition. The most common type of cell was the lymphocyte,—large, small, and intermediate. Almost all the cells in the fluid might be of this type. Large mononuclear cells with cytoplasm that was relatively more basophilic and a regular or an irregular or sometimes even a lobed nucleus were always present and might account for at least half the cells. Polymorphonuclear cells were usually absent, but were present in all the fluids obtained on the second day of the disease and in 80 per cent. of those obtained on the third day. The average differential count of these cells was 34 per cent. on the second day and 6 per cent. on the third. Endothelial cells like those seen in smears from the surface of the meninges were rare. In every fluid degenerating cells were found that showed no cytoplasm or only ragged shreds of cytoplasm and a nucleus that was breaking down. These cells were probably mononuclear in type, but classification was usually impossible. They were present in some fluids to the extent of 20 to 30 per cent. A few plasma cells could also be identified in some of the fluids. In the more acute stages large cells with very irregular nuclei and a faintly basophilic and vacuolated cytoplasm were present in considerable numbers. Large phagocytic cells were very rare. Accurate differential counts were not attempted in all cases, for an exact classification of the cells was difficult, every grade of transition from one type of mononuclear to another being found. In the examinations made, the following were the maximal and minimal proportions of each type:

Polymorphonuclear	0 to 85 per cent.
Lymphocytes (large, small, intermediate)	17 to 89 per cent.
Endothelium of meninges	0 to 6 per cent.
Other types of mononuclear cells	3 to 70 per cent.
Degenerating cells	4 to 24 per cent.

The cases that showed many polymorphonuclear cells in the early stages later showed the usual mononuclear excess.

Lumbar puncture was performed in ten cases that were either in the preparalytic stage or that never developed definite paretic symptoms. The fluids from two children seen on the second day after onset of symptoms contained 880 and 113 cells per cubic millimeter, and they developed typical paralyses one and two days later, respectively. The fluid from one case seen on the fourth day of symptoms contained 650 cells per cubic millimeter and gave a + globulin reaction. The child developed respiratory paralysis three days later and died. Another case seen on the sixth day, with a fluid containing thirty-seven cells per cubic millimeter, developed paralysis two days later. The spinal fluids of six abortive cases were examined and five of them contained an increase in the number of cells, the number varying from thirty-four to eighty-nine per cubic millimeter. The sixth abortive case was that of a child who suffered from fever and irritability, with tenderness and slight stiffness of the neck, and who was living in the same house with a child who, a few days later, developed typical paralysis. Lumbar puncture was performed on two occasions, the fourth and tenth days after onset of symptoms, but on both occasions the fluid showed no abnormalities. From one of the abortive cases a normal fluid was obtained on the second day after onset of the symptoms, and a fluid containing sixty-two cells per cubic millimeter on the following day. The large proportion of cases that gave abnormal fluids early in the disease is represented in text-figure 2, where the number of fluids abnormal in either cell count, globulin content, or both, examined on each day of the disease, is represented.

From this figure it is seen that nearly every fluid obtained during the first week after onset of symptoms was abnormal. During the second week the number of abnormal fluids diminished slightly, and at the beginning of the third week the percentage dropped to 62



TEXT-FIG. 2. Curve showing per cent. of cases with abnormal cerebrospinal fluids, on each day of the disease in acute poliomyelitis.

per cent. In the second half of the third week the percentage rose again. This rise was due to the number of fluids giving abnormal globulin reactions at this period of the disease. After the fourth week so few fluids were examined on any one day that the daily figures are of little value. From the point of view of estimating the value of lumbar puncture in making an early diagnosis, it is important to know on what day after the onset of symptoms the first signs of paralysis were noted. It was found that paralysis was noted on the same day as the first definite symptom; that is, on the first day of the disease in five cases, on the second day in thirty cases, on the third in thirty-two cases, on the fourth in sixteen cases, on the fifth in fourteen cases, on the sixth in seven cases, on the seventh in two cases, on the eighth in four cases, on the tenth in one case, and on the fifteenth in one case. In most cases, then, paralysis developed after two or more days of preparalytic symptoms. Of the spinal fluids examined on the second day after onset of symptoms, 86 per cent. were abnormal, and all of those examined on the third day showed definite abnormalities. It would appear, therefore, that in most cases the spinal fluid would give indications of pathological conditions before the onset of paralysis. In tuberculous meningitis and in syphilitic myelitis the clinical picture closely resembles that of acute epidemic poliomyelitis, and the characters of the spinal fluid are similar as regards the number and type of cells present and the character of the globulin reaction. The finding of the tubercle bacillus in the fluid in tuberculous meningitis and a positive Wassermann reaction in the spinal fluid in syphilitic myelitis is a sufficient differentiation, as the Wassermann reaction was performed on the fluids of all the poliomyelitis cases in the hospital in 1912 and was negative in every case.

The relation between the severity of the attack and the changes in the spinal fluid is not capable of accurate determination. While all the cases that died during the acute stages showed marked abnormalities in the number of cells present or in the globulin reaction, comparatively mild or even abortive cases were accompanied by just as marked abnormalities. Nor did the cases that showed the more extensive changes in their spinal fluids always give clinical

signs of more extensive meningeal involvement. The localization of the paralysis did not seem to affect the extent of the changes in the fluids; the cases that showed involvement of the lower limbs and thus of the lower segments of the spinal cord did not consistently show greater changes in the spinal fluid obtained by lumbar puncture, while those cases that showed clinical evidence of lesions confined to the upper part of the cord or to the bulb often showed as great changes in the fluid.

The prognosis as to life depends on the localization of the paralysis as well as on the severity, while the prognosis as to ultimate return of muscular power depends rather on the completeness of the damage done to any particular part of the spinal cord. The examination of the spinal fluid does not seem to offer evidence as to the localization or the severity of the damage.

CONCLUSIONS.

The spinal fluid in the cases of acute epidemic poliomyelitis examined was usually clear, colorless, and did not appear to be under any great increase of pressure. It showed changes in the number of cells present, or in the globulin content, or in both, in the majority of cases examined on the first few days after onset of symptoms. The number of cells was usually highest during the first week, and in one case reached the figure of 1,221 per cubic millimeter. The globulin reaction was usually most marked during the third week. The number of cells diminished rapidly and was above normal in only 32 per cent. of the cases in the third week. The increase in the globulin reaction persisted to the fourth week and might be present for a considerably longer period. The cell increase was due almost invariably to mononuclear cells of various types. The lymphocytic type of cell was the most common. A high polymorphonuclear count was noted in the very early stages. All the fluids reduced Fehling's solution. The examination of the spinal fluid may be of value in diagnosis in the preparalytic stages and in abortive cases. It is not of value in prognosis as to life or ultimate recovery.

THE CULTIVATION OF AMŒBÆ IN PURE CULTURE UPON AUTOLYZED TISSUES.*

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The first attempts to propagate amœbæ upon artificial media date from Cunningham's[1] work in 1879, or possibly from Auerback's[2] in 1856. Until the recent work of Williams[3] in 1911 multiplication of these protozoa was obtained only in association with living bacteria, as the earlier reports of Kartulis,[4] Casagrandi and Barbagallo,[5] Tsujitani,[6] Lesage,[7] and others, who claim to have grown them in pure culture upon various media, have not been confirmed. While Musgrave and Clegg[8] state that the amœbæ from man and other sources cannot be cultivated without bacteria, they admit that there are indications that the parasites in some liver abscesses grow without them. They contend, however, that these amœbæ have lived at one time in symbiosis with some microörganism. The absence of bacteria in these lesions is explained by the gradual adaptation of the parasite to the tissue environment, to a point where the symbiont is replaced by some other agent, possibly an enzyme or ferment peculiar to the liver.

The studies of Wolbach and Saiki,[9] who found obligate anaerobes in the liver of healthy dogs, might support the contention of Musgrave and Clegg that in the liver of man one or more varieties of bacteria are often present, and that these are responsible for the growth and multiplication of the amœbæ *in vivo*. While bacteria are sometimes found in amœbic abscesses prior to surgical intervention, in our experience this is exceptional. Even in the presence of microörganisms our experiments would seem to indicate that the symbiosis is between amœbæ and tissue and not between amœbæ and bacteria.

The possibility that tissue cells might in some way act as food for amœbæ

* Received for publication, May 16, 1913.

[1] Cunningham, *Quart. Jour. Micr. Sc.*, 1881, xxi, 234.

[2] Auerback, *Ztschr. f. wissensch. Zool.*, 1856, vii, 365.

[3] Williams, A. W., *Jour. Med. Research*, 1911–12, xxv, 263.

[4] Kartulis, *Centralbl. f. Bakteriol., Orig.*, 1890, vii, 54.

[5] Casagrandi, O., and Barbagallo, P., *Centralbl. f. Bakteriol., Orig.*, 1897, xxi, 579.

[6] Tsujitani, J., *Centralbl. f. Bakteriol., Orig.*, 1898, xxiv, 666.

[7] Lesage, A., *Compt. rend. Soc. de Biol.*, 1907, lxii, 1157.

[8] Musgrave, W. E., and Clegg, M. T., *Philippine Islands, Bureau of Government Laboratories Reports*, 1904, No. 18.

[9] Wolbach, S. B., and Saiki, T., *Jour. Med. Research*, 1909, xxi, 267.

had been suggested by Councilman and Lafleur,[10] Lesage,[11] and others, but Williams[12] was the first to adopt the idea that bits of tissue would furnish a suitable medium for the cultivation of these protozoa and replace their symbiotic microörganism. By using methods usually recommended for the separation of amœbæ from bacteria, she succeeded in growing certain species from the intestine of man and some of the lower animals upon neutral agar plates smeared with bits of sterile brain of the guinea pig, rabbit, and dog. Further than to describe the technique of preparing the medium, the source of the amœbæ, and the possibility that pure cultures offer to further study, Williams attempts no explanation of the principles involved.

Since we have found by repeating the experiments of Williams[13] that disintegration of the tissue bits is followed by growth of the amœbæ and that no growth takes place as long as the tissues are fresh, we thought that autolysis was responsible for her success, and, if so, that better results should follow the use of previously autolyzed tissue for the cultivation of the amœbæ from liver abscesses.

METHOD OF CULTIVATION.

Bits of sterile liver, kidney, and brain of healthy rabbits, guinea pigs, kittens, and human placenta are collected in sterile flasks, sealed, and placed in a thermostat at 40° C. for ten to twenty days. While a longer time is required for complete autolysis there is sufficient disintegration and liquefaction in the shorter period to render the material suitable for use.

The fluid tissue titrates from 1 to 10 per cent. acid against phenolphthalein, depending upon the length of time that disintegration is allowed to continue. A neutral or slightly acid reaction (not exceeding 1.5 per cent. acid) has given the best results. Should the reaction of the autolyzed tissues exceed this point correction may be made in the usual way by the addition of normal sodium hydroxide.

By means of a sterile pipette 0.1 to 0.5 of a cubic centimeter of autolyzed tissue is distributed over the surface of a neutral agar slant and the tube kept for several hours in the horizontal posi-

[10] Councilman, W. T., and Lafleur, H. A., *Johns Hopkins Hosp. Rep.*, 1891, ii, 395.

[11] Lesage, A., *loc. cit.*

[12] Williams, A. W., *loc. cit.*

[13] Williams, A. W., *loc. cit.*

tion to permit absorption of the tissue juice by the agar. The tube may then be stood upright, incubated to determine its sterility, and is ready for use.

Equally good results may be obtained if the agar is previously melted and the tissue juice added to it, but care should be taken that the temperature of the agar does not exceed 50° C. and the surface of such slants should be broken before using; for the tissue fragments quickly gravitate when the tubes are slanted and allowed to cool, and the amœbæ are unable to obtain from the surface of the agar a sufficient amount of the autolyzed material for their nourishment. Our earlier failure to cultivate amœbæ from a bacteria-free liver abscess upon autolyzed tissue prepared in this manner is, we believe, accounted for by neglecting to recognize this important point.

The agar upon which the autolyzed tissues are smeared is made in the usual way and neutral in reaction. While it has been noted that better growth is obtained upon a neutral agar base, good results follow if the reaction of the agar does not exceed 0.5 alkalinity. Upon an acid agar base it has not been possible to induce the multiplication of the amœbæ. Since the tissue juices are the essential factors in the cultivation of the protozoa without bacteria, the addition of nutritious substances (peptones, beef extract, etc.) to the agar is unnecessary as these substances are of no value in the development of the protozoa without bacterial symbionts.

With these tissue juices we have cultivated an amœba from a liver abscess. We were unfortunate in that the material from this abscess proved not to be sterile and, for the present, still leaves the problem in doubt. But the comparative ease with which the amœbæ were separated from the bacteria present, with the knowledge that cultures made from the fluid aspirated from the liver previous to surgical interference were sterile, leaves little doubt but that the symbiosis was between amœbæ and tissue and not between amœbæ and bacteria.

In like manner five species of amœbæ isolated by one of us (Couret) from the intestine in frank amœbic dysentery and growing in symbiosis with bacteria, and a culture of amœba with *Bacillus typhosus* from Musgrave and Clegg have been separated from their

associated microörganisms and have now been growing for several generations on autolyzed tissue of the liver, kidney, and brain of rabbits and guinea pigs, and upon autolyzed human placenta and autolyzed defibrinated blood.

The necessity of using autolyzed rather than fresh bits of tissues in the cultivation of amœbæ from bacteria-free liver abscesses is evident from what is known to occur to these parasites when removed from their favorable environment in the liver. Active amœbæ in liver pus disintegrate and disappear completely in less than twenty-four hours under the most favorable conditions of moisture and temperature. We are not at present prepared to explain why this occurs, or why the active parasites should not encyst or sporulate, but the scarcity or absence of either active or encysted amœbæ or their spores after the lapse of such a short time suggests that some form of ferment action, whether autogenous or heterogenous, destroys the active amœbæ before they can protect themselves. If the medium should not be fully prepared to continue without interruption the growth and multiplication of the active amœbæ within twenty-four hours after the material is planted, little success can be hoped for, since the few scattered encysted forms and spores present, for some reason not yet understood, fail to leave their cyst when transferred to any medium that we have so far employed.

Upon this medium, multiplication of amœbæ previously grown on tissue bits is abundant in twenty-four hours at room or incubator temperature. But with amœbæ cultivated from the liver abscess or in cultures recently separated from their symbiotic bacterium growth is scant in the thermostat and more so at room temperature, for several generations, though frequent transplants (three to seven days) appear to increase the multiplication of the parasites.

The addition of heated (40° to 50° C.), unheated, or filtered serum of an animal to its own tissue juices has apparently been of little advantage in increasing the growth of amœbæ; neither has the addition of 0.5 per cent. dextrose and glycocoll exerted any noticeable effects upon the culture. It is possible that increased or decreased amounts of these substances may in some manner influence the early growths.

No attempt has been made in the present study to determine whether anaerobic conditions would influence the initial growth or subsequent transplants of amœbæ from the liver. Previous attempts with amœbæ from a sterile liver abscess upon a medium composed of human serum heated at 40° C. for one hour with washed human corpuscles and dextrose and glycocoll were unsuccessful, although in the same medium under aerobic conditions the amœbæ were found still active at the end of seven days.

DISCUSSION AND CONCLUSIONS.

Since the purpose of this paper is to record the cultivation of amœbæ upon autolyzed tissue without bacterial association, the morphological characteristics, life cycle, means of differentiating species, and pathogenicity of the protozoa have been omitted. These subjects will be considered in later publications.

The result of this study proves that some species of amœbæ from liver abscesses and the human intestine can be cultivated upon various autolyzed tissues of man and some of the lower animals without a symbiotic microörganism. Their cultivation from liver abscesses upon such bacteria-free autolyzed tissue indicates that their multiplication in these lesions depends upon some product or products in the process of dissociation of the liver cells. That such a process exists in amœbic liver abscesses cannot be questioned when histological and biochemical studies are made of such lesions, and this explains not only why the multiplication of the parasites in the organ occurs, but suggests the probable origin of the lesion.

It has long been known that tissue kept for several days in a perfectly aseptic condition and at body heat, or preferably at slightly higher temperature undergoes softening and final disintegration of its cells. Wells[14] and others who have made a thorough study of this phenomenon find that different enzymic actions take place in this process: thus in the liver they find that soluble nitrogen compounds are greatly increased, the nucleoproteids are altered by nuclease, the purin bases are freed and in their turn acted upon by the guanase and adenase, the fats are split and fatty acids set free,

[14] Wells, G. H., Chemical Pathology, Philadelphia, 1907.

the glycogen gives rise to glucose and undergoes further splitting. lecithin is cleaved, and allied bodies appear, and there is a marked appearance of cholin and cholesterin. Similar changes varying only in degree occur in the process of autolysis of other tissues.

Furthermore, Duval[15] in his experiments upon the cultivation of *Bacillus leprœ* found that the initial multiplication was accomplished when the specific organism was in symbiosis with an associated bacterium capable of hydrolyzing the leprous tissue. In later experiments he noted that the products of split proteins supply what is actually required for the growth of this particular obligate cell parasite, and that while this end is reached with bacteria through their proteolytic action equally good results can be obtained with tissue free from contaminating microörganisms provided that it is allowed to autolyze.

The separation of amœbæ cultivated from the human intestine from their bacterial symbiont, and their development upon various autolyzed tissues indicate that it is not the bacterium that is essential for the life of these protozoa, but the action of the living bacteria upon the protein contained in the media. This would explain the failure of many investigators to cultivate amœbæ with dead bacteria or bacterial filtrates.

Mention has been made that the autolyzed tissue used in the cultivation experiments gave a distinct acid reaction. The multiplication of amœbæ upon a medium with such a reaction appears contradictory to the findings of Musgrave and Clegg,[16] Walker,[17] and others, who have emphasized the necessity of an alkaline medium for the successful cultivation of amœbæ with a bacterial symbiont, though in accord with what is known to be the reaction of the contents from amœbic liver abscesses and of the bloody stools in intestinal amœbiosis. The fact should not be lost sight of that, in the cultivation of amœbæ, these authors lay stress upon the selectiveness of amœbæ for a special microörganism. A comparison of their work with our own results indicates that bacteria known to possess strong hydrolyzing properties, *e. g., Vibrio cholerœ, Bacillus coli,*

[15] Duval, C. W., *Jour. Exper. Med.*, 1911, xiii, 365.

[16] Musgrave, W. E., and Clegg, M. T., *loc. cit.*

[17] Walker, E. L., *Jour. Med. Research.*, 1907–08, xvii, 370.

Bacillus subtilis, Vibrio proteus (Finkler-Prior), etc., furnish the best symbionts to the amœbæ. It is well known that these bacteria growing upon gelatin or blood serum liquify the medium and alter its reaction to a marked degree of acidity. This acidity, for the most part, results from dissociation of the protein molecule contained in the media, for Wiener[18] has shown that autolysis does not begin until the normal alkalinity of the tissues has been neutralized by the production of organic acids.

Since experiments show that the development of the amœbæ is scant or completely arrested if the autolyzed tissue is smeared upon an acid agar base or upon agar with an alkalinity higher than 1 per cent., the limit within which multiplication can occur must be small. It is possible that this explains why amœbæ are so few in the center of liver abscesses where the acidity is very high and so plentiful in or near its walls where the normal tissue juices furnish a controlling influence over the acidity of the autolyzing tissue. It explains also why a neutral or a 1 to 1.5 per cent. alkaline medium is essential for the cultivation of amœbæ with a bacterial symbiont. Here the necessity for a medium with an alkaline reaction seems necessary, as bacteria, especially those having high hydrolyzing properties, develop a marked acidity in the medium. If the medium possesses an initial acidity the limit is either quickly reached or already present and no multiplication occurs. On the other hand, a medium with a reaction too alkaline either inhibits the growth of symbiotic microörganisms, or neutralizes the acid products as rapidly as they are formed by the associated bacterium.

Whether the amœbæ cultivated from liver abscesses and from the intestinal canal upon diverse autolyzed tissues are able to produce lesions similar to those from which they were isolated, or whether they are non-pathogenic species which are accidental contaminators to those responsible for frank lesions, remains still to be determined. Experiments bearing upon these points, as well as on many others made possible by this work, are now under way.

[18] Wiener, cited by Adami, J. G., The Principles of Pathology, Philadelphia and New York, 1908, i, 338.

ON THE EXCRETION OF GLYCURONIC ACID IN PNEUMOCOCCUS INFECTIONS, WITH SPECIAL REFERENCE TO LOBAR PNEUMONIA.*

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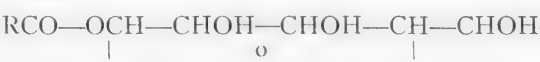
The present problem was suggested by the fact that glycuronic acid has the property of combining in the living animal organism with a great many chemically well defined organic substances, which may be either products occurring under normal conditions in the animal body, or drugs or other chemicals foreign to the animal organism.[1] They belong to almost all chemical groups. It is generally admitted that after the reaction has taken place in the organism and the double compounds have been formed, they are excreted in the urine without further transformation.

There are two types of chemical combination that have been recognized in these compounds: (1) the glucosidic type,

demonstrated for the first time by Fisher and Piloty,[2] and (2) the ester type,



discovered by Jaffé.[3] It is thus obvious that the chemical functions that react with the aldehyde group of glycuronic acid are alcohol, phenol, and acid. The substances that do not possess these chemical groups have to undergo certain changes before they become suitable for reaction.

* Received for publication, May 19, 1913.

[1] A complete review of the subject is given by Neuberg, C., Der Harn, sowie die übrigen Ausscheidungen und Körperflüssigkeiten, Berlin, 1911, i, 429.

[2] Fisher, E., and Piloty, O., *Ber. d. deutsch. chem. Gesellsch.*, 1891, xxiv, 522.

[3] Jaffé, M., *Ztschr. f. physiol. Chem.*, 1904–5, xliii, 374.

Since a great many of the substances that are excreted in the urine, combined with glycuronic acid, have a marked toxic effect on the animal organism, this acid is generally regarded as a detoxifying agent, as are also sulphuric acid and glycocoll. Mayer and Neuberg found that glycuronic acid is a constant ingredient of the normal urine of man and other mammals.[4] This acid is always excreted in a conjugated form, and, according to these authors, the conjugated bodies are phenol and indoxyl.

The present paper deals with the excretion in the urine of glycuronic acid during pneumococcus lobar pneumonia, and a few other pulmonary or pleural febrile affections. Its excretion by rabbits with general pneumococcus infections has also been studied.[5] Quantitative relations merely are considered. No attempt has been made to estimate and identify the conjugated bodies.

METHOD.

All estimations have been made according to the method of B. Tollens and his co-workers,[6] as modified for urine by C. Tollens.[7] This method gives better results than the ones formerly used (the p-bromphenylhydrazine and the polarimetric methods). It is based on the fact that glycuronic acid, in a free as well as in a conjugated state, yields furfurol by boiling with hydrochloric acid. The furfurol may be distilled over with the hydrochloric acid and precipitated with phloroglucin. The amount of furfurol-phloroglucid precipitate multiplied by three represents the original quantity of glycuronic acid.

The directions given by Tollens have been followed with but slight modifications. 250 cubic centimeters of fresh urine (the twenty-four hour specimen of a rabbit) are first precipitated

[4] Mayer, P., and Neuberg, C., *Ztschr. f. physiol. Chem.*, 1900, xxix, 256.

[5] The excretion of glycuronic acid in disease (chiefly in diabetes and dyspneic conditions) has been studied by various investigators (Neuberg, *loc. cit.*). Their figures, however, cannot be used for comparison with those obtained in this work, as they used the polarimetric method of estimation, and it was found that the results do not coincide.

[6] Lefevre, K. U., and Tollens, B., *Ber. d. deutsch. chem. Gesellsch.*, 1907, xl, 4513.

[7] Tollens, C., *Ztschr. f. physiol. Chem.*, 1909, lxi, 95; 1910, lxvii, 138; Tollens, C., and Stern, F., *ibid.*, 1910, lxiv, 39.

with five cubic centimeters of ammonia and with basic lead acetate solution until all precipitable substances are thrown down. An excess of reagent must be carefully avoided. After standing for at least sixteen hours, the precipitate is separated from the liquid by centrifugalization and thoroughly washed three times with distilled water in 100 cubic centimeter tubes.[8] If necessary, a few drops of ammonia are added to each washing to prevent the solution of the precipitate. The precipitate is then brought quantitatively into a flask of one liter capacity, 12 per cent. hydrochloric acid being used for rinsing the tubes, and subjected to distillation in an apparatus similar to the arrangement used for distilling ammonia in the Kjehldahl process. The flask contains first about 120 cubic centimeters of precipitate and hydrochloric acid. After distilling over sixty cubic centimeters of solution, a fresh amount of sixty cubic centimeters of 12 per cent. hydrochloric acid is added to the flask and the same operation repeated until 600 cubic centimeters of distillate are obtained.

The distilled solution is then filtered and 0.25 of a gram of phloroglucin dissolved in 12 per cent. hydrochloric acid is added. In order to determine whether or not all the amount of furfurol has distilled over, about 100 cubic centimeters more of distillate are collected in a separate beaker and the amount of furfurol present is tested by adding a little phloroglucin solution.[9] The distillation is continued until phloroglucin no longer precipitates. An amount of 600 cubic centimeters of distillate, however, has been found sufficient in almost all cases. The furfurol-phloroglucid is allowed to stand for at least sixteen hours, is then collected on a Gooch filter, thoroughly washed with 150 cubic centimeters of distilled water, and dried to constant weight at 100° C., the crucibles being kept in closed, glass stoppered weighing bottles, in order to avoid the rapid absorption of moisture by the precipitate. In calculating the total amount of furfurol-phloroglucid, a correction for the quantity remaining in solution

[8] No filter paper is used. The usual correction for the amount of furfurol developed by filter paper when boiling with hydrochloric acid thus becomes superfluous.

[9] This procedure was preferred to the color test with anilin acetate used by Tollens.

must be made. This is 0.0092 of a gram per liter of distillate, as given by Tollens.

About sixty estimations have been made on normal individuals and on non-febrile syphilitic patients, without gastro-intestinal disturbances. According to Tollens, a normal adult on a mixed diet usually excretes 0.3 to 0.4 of a gram of glycuronic acid in twenty-four hours. The figures are slightly higher when the diet is rich in meat and fat. Occasionally the excretion may be much larger (up to 0.82 of a gram) without giving any evidence of a pathological process.

The findings of Tollens have been generally confirmed. The variations, however, from day to day and with the change in diet have been more frequent and larger than in the cases of Tollens. Normal persons and non-febrile syphilitic patients weighing fifty-five to seventy kilograms, on a mixed diet composed of milk, bread, vegetables, fruits, and moderate amounts of meat, excreted daily 0.4 to 0.6 of a gram of glycuronic acid. Not infrequently, however, with a large intake, the quantities of glycuronic acid excreted were as high as 0.8 and even 0.95 of a gram. For instance, H. B. (table III), a white male patient convalescing from pneumonia, weighing fifty-five kilograms, excreted daily 0.9 and 0.93 of a gram on a rich diet of milk, soup, bread, butter, cocoa, coffee, steak, roast beef, spinach, cereals, string beans, rice pudding, eggs, and prunes. Eight days later the average output of the same patient was only 0.6 of a gram of glycuronic acid, the diet being composed of milk, bread, butter, and small amounts of chicken and beef.

The results have been cited in order to show that in judging the significance of the quantity of glycuronic acid excreted in the cases that follow (tables I to XIII), the quality as well as the quantity of the intake must be considered. The analyses are from patients who did not show an appreciable gastro-intestinal disturbance during the disease. The urine contained only traces of albumin even during the high febrile period, and in no phase of the disease was there present a demonstrable amount of sugar (tested by the reduction of Fehling's solution).

TABLE I.

R. F., White Male Patient, Age Thirty-Five Years. Weight Seventy Kilograms. Diagnosis: Pneumonia, Lobar; Consolidation of Left Lower Lobe; Transitory Septicemia and Pleurisy, Serofibrinous.

Date.	Day of disease.	Urine in c.c. / Specific gravity at 15° C.	Furfurol-phloroglucid in gm.	Glucuron in gm.	Rectal temperature, C. Maximum. Minimum.	Intake and remarks.
1912 Nov. 18	6	1,350 / 1,023	0.404	1.212	41.5° 37.3°	*Drugs:* codein 64 mg., morphia 11 mg. *Food:* water 2,800 c.c., milk 1,050 c.c., orange juice 340 c.c., albumen 80 c.c., cream soup 150 c.c., eggs 1, rice 30 gm. *White blood cells:* 16,000.
Nov. 19	7	1,380 / 1,025	0.392	1.176	41.3° 37.0°	*Drugs:* codein 64 mg., morphia 11 mg. *Food:* water 3,000 c.c., milk 1,300 c.c., soup 200 c.c., cereal 30 gm., eggs 2, potatoes 30 gm., rice 45 gm., toast 1 piece.
Nov. 20	8	1,340 / 1,019	0.324	0.972	38.0° 37.0°	*Drugs:* none. *Food:* water 2,700 c.c., milk 1,100 c.c., orange juice 70 c.c., cream soup 200 c.c., albumen 30 c.c., cereal 75 gm., eggs 3, rice 30 gm., toast 3 pieces, tapioca pudding 45 gm., sugar 10 gm., potatoes 45 gm.
Nov. 21	9	1,610 / 1,018	0.340	1.020	37.5° 37.0°	*Drugs:* none. *Food:* water 1,500 c.c., milk 1,100 c.c., orange juice 35 c.c., cream soup 100 c.c., cereal 75 gm., eggs 2, toast 3 pieces, potatoes 45 gm., albumen 25 c.c., rice 45 gm.
Nov. 22	10	1,490 / 1,023	0.325	0.975	37.5° 37.3°	*Drugs:* codein 32 mg. *Food:* water 2,200 c.c., milk 1,000 c.c., orange juice 160 c.c., cream soup 150 c.c., albumen 30 c.c., cereal 75 gm., eggs 2, toast 2 pieces, potatoes 30 gm., sugar 20 gm.
Nov. 23	11	1,360 / 1,020	0.335	1.005	37.6° 37.0°	*Drugs:* codein 32 mg. *Food:* water 1,500 c.c., milk 1,200 c.c., orange juice 220 c.c., albumen 50 c.c., cereal 60 gm., eggs 2, toast 3 pieces, custard 45 gm., potatoes 35 gm., crackers 2 pieces, baked apple 1.
Nov. 24	12	1,410 / 1,023	0.288	0.864	37.5° 37.2°	*Drugs:* codein 32 mg. *Food:* water 1,000 c.c., milk 1,400 c.c., orange juice 220 c.c., albumen 70 c.c., cereal 130 gm., eggs 2, toast 5 pieces, soup 250 c.c., potatoes 45 gm., spinach 30 gm., sugar 20 gm., pudding 30 gm.
Nov. 25	13	940 / 1,026	0.237	0.711	37.5° 36.8°	*Drugs:* codein 32 mg. *Food:* water 1,300 c.c., milk 1,000 c.c., orange juice 250 c.c., albumen 25 c.c., soup 150 c.c., cream 50 c.c., ice cream 45 gm., eggs 4, toast 3 pieces, pudding 30 gm., cereal 45 gm.

TABLE I.—*Concluded.*

Date.	Day of disease.	Urine in c.c. / Specific gravity at 15° C.	Furfurol-phloroglucid in gm.	Glucuron in gm.	Rectal temperature, C. Maximum. / Minimum.	Intake and remarks.
1912 Nov. 26	14	1,100 / 1,028	0.266	0.798	37.5° / 37.0°	*Drugs:* codein 32 mg. *Food:* water 1,500 c.c., milk 1,000 c.c., orange juice 250 c.c., albumen 25 c.c., soup 250 c.c., cream 50 c.c., rice 75 gm., potatoes 15 gm., bread 4 slices, pudding 15 gm., eggs 3, cereal 30 gm.
Nov. 27	15	950 / 1,027	0.227	0.681	37.5° / 36.7°	*Drugs:* none. *Food:* water 1,800 c.c., milk 1,000 c.c., orange juice 250 c.c., albumen 25 c.c., soup 250 c.c., creamed celery 60 gm., toast 7 pieces, cocoa 250 c.c., eggs 4, cereal 60 gm.
Dec. 13	31	1,150 / 1,025	0.284	0.852	37.5° / 37.0°	*Drugs:* none. *Food:* copious mixed diet with meat and fruits.
Dec. 14	32	1,400 / 1,027	0.279	0.837	37.4° / 36.6°	*Drugs:* none. *Food:* copious mixed diet with meat and fruits. Patient recovered and was discharged 5 days later.

TABLE II.

M. B., White Female Patient, Age Eighteen Years, Weight Forty-Five Kilograms. Diagnosis: Pneumonia, Lobar; Involvement of Right Upper Lobe.

Date.	Day of disease.	Urine in c.c. / Specific gravity at 15° C.	Furfurol-phloroglucid in gm.	Glucuron in gm.	Rectal temperature, C. Maximum. Minimum.	Intake and remarks.
1912						
Nov. 19	4	1,570 / 1,012	0.353	1.059	39.5° / 38.7°	*Drugs:* none. *Food:* water 1,300 c.c., milk 520 c.c., orange juice 310 c.c., lemon juice 12 c.c., albumen 120 c.c., sugar 20 gm. *White blood cells:* 46,000.
Nov. 20	5	1,630 / 1,019	0.295	0.885	39.9° / 39.3°	*Drugs:* codein 96 mg. *Food:* water 1,700 c.c., milk 750 c.c., orange juice 250 c.c., milk soup 150 c.c., albumen 85 c.c.
Nov. 21	6	1,390 / 1,012	0.314	0.942	39.9° / 39.0°	*Drugs:* morphia 24 mg. *Food:* water 1,600 c.c., milk 900 c.c., grape juice 100 c.c., orange juice 40 c.c., albumen 60 c.c., sugar 10 gm.
Nov. 22	7	1,870 / 1,019	0.279	0.837	39.9° / 38.5°	*Drugs:* codein 96 mg. *Food:* water 1,400 c.c., milk 1,500 c.c., orange juice 200 c.c., albumen 70 c.c., sugar 15 gm.
Nov. 23	8	2,130 / 1,009	0.351	1.053	38.5° / 37.3°	*Drugs:* codein 64 mg. *Food:* water 1,400 c.c., milk 800 c.c., orange juice 270 c.c., albumen 75 c.c., sugar 20 gm.
Nov. 24	9	1,080 / 1,017	0.251	0.753	37.4° / 37.3°	*Drugs:* none. *Food:* water 1,000 c.c., milk 850 c.c., orange juice 270 c.c., albumen 75 c.c., sugar 20 gm.
Nov. 25	10	830 / 1,022	0.244	0.732	37.7° / 37.3°	*Drugs:* none. *Food:* water 700 c.c., milk 860 c.c., orange juice 210 c.c., albumen 45 c.c., eggs 1, ice cream 75 gm., rice 60 gm., tapioca 45 gm., toast 3 pieces.
Nov. 26	11	1,380 / 1,016	0.279	0.837	37.3° / 37.0°	*Drugs:* none. *Food:* water 700 c.c., milk 300 c.c., orange juice 110 c.c., cream soup 200 c.c., albumen 25 c.c., cocoa 150 c.c., bread 1 slice, cereal 75 gm., eggs 1, toast 2 pieces, potatoes 30 gm.
Nov. 27	12	1,150 / 1,020	0.230	0.690	37.2° / 36.5°	*Drugs:* none. *Food:* water 900 c.c., milk 800 c.c., cocoa 250 c.c., orange juice 200 c.c., cream soup 200 c.c., cereal 60 gm., albumen 25 c.c., eggs 1, bread 7 slices, creamed celery 20 gm., rice custard 45 gm., pudding 30 gm.
Dec. 4	19	900 / 1,026	0.289	0.867	37.1° / 36.3°	*Drugs:* none. *Food:* copious mixed diet, meat and fruits included.
Dec. 5	20	1,010 / 1,022	0.249	0.747	37.3° / 36.5°	*Drugs:* none. *Food:* copious mixed diet, meat and fruits included. Patient recovered. She was discharged 4 days later.

TABLE III.

H. B., White Male Patient, Age Twenty-Two Years, Weight Fifty-Five Kilograms.

Diagnosis: Pneumonia, Lobar (Relapse); Involvement of Right Upper and Lower Lobes.

Date.	Day of disease.	Urine in c.c. / Specific gravity at 15° C.	Furfurol-phloroglucid in gm.	Glucuron in gm.	Rectal temperature, C. Maximum. Minimum.	Intake and remarks.
1912						
Dec. 9	16	2,080 / 1,014	0.400	1.200	40.5° 39.6°	*Drugs:* codein 32 mg., morphia 16 mg. *Food:* lactose 360 gm., water 4,000 c.c., milk 950 c.c., orange juice 700 c.c., lemon juice 100 c.c., eggs 1, albumen 50 c.c.
Dec. 10	17	1,470 / 1,017	0.435	1.305	40.3° 39.6°	*Drugs:* codein 32 mg., morphia 16 mg. *Food:* lactose 420 gm., water 2,600 c.c., milk 130 c.c., orange juice 550 c.c., cocoa 150 c.c., cream soup 250 c.c., albumen 20 c.c.
Dec. 11	18	3,350 / —	0.670	2.010	40.3° 40.0°	*Drugs:* morphia 48 mg., caffein sodium salicylate 160 mg., camphor oil 400 mg. *Food:* lactose 360 gm., water 2,300 c.c., milk 1,900 c.c., orange juice 450 c.c., albumen 10 c.c., cream soup 200 c.c.
Dec. 12	19	1,770 / 1,015	0.457	1.371	40.0° 38.5°	*Drugs:* morphia 32 mg., caffein sodium salicylate 224 mg., camphor oil 500 mg. *Food:* lactose 300 gm., water 2,300 c.c., milk 1,400 c.c., orange juice 350 c.c., cocoa 750 c.c., lemon juice 20 c.c., albumen 30 c.c.
Dec. 13	20	1,590 / 1,015	0.468	1.404	38.5° 36.8°	*Drugs:* codein 32 mg., caffein sodium salicylate 160 mg., camphor oil 100 mg. *Food:* lactose 200 gm., water 1,400 c.c., milk 1,400 c.c., orange juice 550 c.c., cocoa 400 c.c., custard 30 gm., albumen 30 c.c.
Dec. 14	21	970 / 1,024	0.351	1.053	37.5° 36.2°	*Drugs:* caffein sodium salicylate 32 mg., morphia 16 mg. *Food:* lactose 40 gm., water 1,400 c.c., milk 1,000 c.c., orange juice 450 c.c., coffee 100 c.c., cocoa 200 c.c., eggs 1, albumen 30 c.c., custard 30 gm., bread 1 piece.
Dec. 31	38	940 / 1,021	0.302	0.906	37.2° 36.8°	*Food:* copious mixed diet,—milk, soup, cocoa, coffee, meat, vegetables, fruits, eggs.
1913						
Jan. 1	39	1,180 / 1,024	0.312	0.936	37.3° 36.8°	*Food:* copious mixed diet,—milk, soup, cocoa, coffee, meat, vegetables, fruits, eggs.
Jan. 9–10	47–48	1,080 / 1,028	0.200	0.600	37.0° 36.5°	Average amounts of two days. *Food:* moderate quantity of milk, bread, chicken, and beef. Patient recovered. He was discharged on Jan. 10.

TABLE IV.

C. F., Colored Male Patient, Age Thirty-Seven Years, Weight Sixty-Three Kilograms.

Diagnosis: Pneumonia, Lobar; Consolidation of Right Lower Lobe.

Date.	Day of disease.	Urine in c.c. / Specific gravity at 15° C.	Furfurol-phloroglucid in gm.	Glucuron in gm.	Rectal temperature, C. Maximum. Minimum.	Intake and remarks.
1912						
Dec. 11	5	780 / 1,025	0.254	0.762	40.5°	*Drugs:* codein 32 mg.
					39.3°	*Food:* lactose 440 gm., water 3,300 c.c., milk 1,800 c.c., orange juice 75 c.c.
Dec. 12	6	1,190 / 1,021	0.302	0.906	40.0°	*Drugs:* none.
					37.3°	*Food:* lactose 400 gm., water 4,600 c.c., milk 1,800 c.c., orange juice 300 c.c., cocoa 150 c.c.
Dec. 13	7	1,100 / 1,018	0.219	0.657	37.3°	*Drugs:* codein 32 mg.
					36.8°	*Food:* lactose 240 gm., water 2,800 c.c., milk 1,000 c.c., orange juice 100 c.c., soup 150 c.c., albumen 20 c.c., custard 30 gm., bread 1 slice.
Dec. 14	8	1,270 / 1,016	0.262	0.786	37.5°	*Drugs:* none.
					37.0°	*Food:* water 1,700 c.c., milk 1,000 c.c., orange juice 200 c.c., custard 45 gm., albumen 30 c.c., bread 2 slices.
1913						
Jan. 8	33	790 / 1,027	0.208	0.624	37.5°	*Food:* mixed diet,—milk, meat, bread, small amounts of vegetables.
					36.7°	
Jan. 9	34	1,010 / 1,022	0.193	0.579	37.0°	*Food:* mixed diet,—milk, meat, bread, small amounts of vegetables.
					36.3°	Patient recovered. He was discharged 2 days later.

TABLE V.

F. R., White Female Patient, Age Twenty-Nine Years, Weight Forty-Five Kilograms.

Diagnosis: Pneumonia, Lobar; Involvement of Right Lower Lobe.

Date.	Day of disease.	Urine in c.c. / Specific gravity at 15° C.	Furfurol-phloroglucid in gm.	Glucuron in gm.	Rectal temperature, C. Maximum. Minimum.	Intake and remarks.
1912						
Dec. 11	8	1,750 / 1,010	0.291	0.873	40.3° / 38.0°	*Drugs:* codein 32 mg. *Food:* water 1,600 c.c., milk 550 c.c., orange juice 170 c.c., cocoa 200 c.c., bread 1 slice, custard 30 gm. *White blood cells:* 16,000.
Dec. 12	9	1,410 / 1,012	0.242	0.726	38.0° / 37.0°	*Drugs:* none. *Food:* water 1,000 c.c., milk 550 c.c., tea 200 c.c., coffee 200 c.c., cocoa 300 c.c., orange juice 150 c.c., toast 1 piece, eggs 2, prunes 4, sugar 10 gm.
Dec. 13	10	910 / 1,016	0.228	0.684	37.0° / 36.7°	*Drugs:* none. *Food:* water 1,000 c.c., milk 450 c.c., orange juice 50 c.c., soup 200 c.c., cocoa 150 c.c., tea 150 c.c., eggs 2, pudding 90 gm., bread and toast 3 pieces, chicken 50 gm.
Dec. 20	17	1,360 / 1,017	0.230	0.690	36.7° / 36.5°	*Food:* copious mixed diet, including meat and fruits.
Dec. 21	18	1,010 / 1,019	0.248	0.744	36.7°	*Food:* copious mixed diet, including meat and fruits. Patient recovered. She was discharged on Dec. 28.

TABLE VI.

A. H., Colored Male Patient, Age Six Years, Weight Twenty Kilograms.

Diagnosis: Pneumonia, Lobar; Involvement of Left Lower Lobe.

Date.	Day of disease.	Urine in c.c. / Specific gravity at 15° C.	Furfurol-phloroglucid in gm.	Glucuron in gm.	Rectal temperature, C. Maximum. Minimum.	Intake and remarks.
1912						
Dec. 21	5	750 / 1,013	0.122	0.366	40.0°	*Drugs:* none.
					39.4°	*Food:* water 1,300 c.c., milk 250 c.c., orange juice 160 c.c., albumen 50 c.c., cocoa 150 c.c., sugar 10 gm.
						White blood cells: 23,000.
Dec. 22	6	870 / 1,009	0.120	0.360	39.6°	*Drugs:* none.
					37.0°	*Food:* water 700 c.c., milk 300 c.c., orange juice 270 c.c., albumen 50 c.c., cocoa 350 c.c., lemon juice 20 c.c.
Dec. 23	7	390 / 1,010	0.070	0.210	37.5°	*Drugs:* castor oil 15 c.c.
					37.2°	*Food:* water 600 c.c., milk 770 c.c., orange juice 260 c.c., albumen 50 c.c., rice 45 gm., bread 4 slices, toast 3 slices, custard 45 gm., lemon juice 50 c.c.
Dec. 24	8	950 / 1,012	0.146	0.438	37.3°	*Drugs:* codein 10 mg.
					37.2°	*Food:* Water 860 c.c., milk 800 c.c., orange juice 150 c.c., bread 4 slices, soup 200 c.c., rice 30 gms., cocoa 200 c.c., eggs 2.
Dec. 25	9	1,150 / 1,014	0.163	0.489	37.4°	*Drugs:* none.
					37.0°	*Food:* water 800 c.c., milk 850 c.c., prunes 8, orange juice 100 c.c., soup 200 c.c., bread 4 slices, rice 15 gm., coffee jelly 30 gm., eggs 2, albumen 20 c.c.
Dec. 31	15	980 / 1,014	0.195	0.585	37.7°	*Food:* copious mixed diet, including meat
					37.0°	and fruits.
1913						
Jan. 1	16	1,300 / 1,014	0.192	0.576	37.7°	*Food:* copious mixed diet, including meat
					37.4°	and fruits.
Jan. 8	23	990 / 1,017	0.116	0.348	37.7°	*Food:* mixed diet,—moderate amounts
					37.2°	of fruits and vegetables.
Jan. 9	24	1,130 / 1,013	0.115	0.345	37.6°	*Food:* mixed diet,—moderate amounts of
					37.4°	fruits and vegetables.
						Patient recovered. He was discharged on Jan. 10.

TABLE VII.

M. M., White Male Patient, Age Twenty-Three Years, Weight Fifty-Four Kilograms.

Diagnosis: Pneumonia, Lobar; Involvement of Right Upper and Lower Lobes.

Date.	Day of disease.	Urine in c.c. / Specific gravity at 15° C.	Furfurol-phloroglucid in gm.	Glucuron in gm.	Rectal temperature, C. Maximum. Minimum.	Intake and remarks.
1912 Dec. 18	4	1,020 / 1,024	0.194	0.582	40.5° 40.0°	*Drugs:* morphia 11 mg., codein 10 mg., calomel 64 mg., magnesium sulphate 15 gm. *Food:* water 1,200 c.c., milk 700 c.c., broth 150 c.c., orange juice 200 c.c., cocoa 150 c.c., albumen (egg) 20 c.c. *White blood cells:* 28,000.
Dec. 19	5	1,310 / 1,023	0.261	0.783	40.4° 39.6°	*Drugs:* codein 16 mg. *Food:* water 2,000 c.c., milk 810 c.c., broth 350 c.c., cocoa 300 c.c.
Dec. 20	6	1,480 / 1,023	0.233	0.699	39.6° 38.8°	*Drugs:* codein 16 mg. *Food:* water 1,500 c.c., milk 700 c.c., soup 200 c.c., cocoa 350 c.c.
Dec. 21	7	1,030 / 1,025	0.207	0.621	38.8° 37.7°	*Drugs:* codein 24 mg., morphia 11 mg. *Food:* water 1,300 c.c., milk 450 c.c., orange juice 50 c.c., albumen 20 c.c., sugar 10 gm., toast 2 slices.
Dec. 22	8	950 / 1,026	0.201	0.603	37.8° 37.2°	*Drugs:* codein 16 mg., morphia 11 mg. *Food:* water 700 c.c., milk 500 c.c., cocoa 300 c.c., orange juice 80 c.c., eggs 2, albumen 20 c.c., bread and toast, 4 slices.
Dec. 23	9	1,160 / 1,024	0.237	0.712	38.7° 37.8°	*Drugs:* codein 32 mg. *Food:* water 1,200 c.c., milk 550 c.c., soup 200 c.c., orange juice 150 c.c., cocoa 300 c.c., custard 25 gm., eggs 2, toast 2 pieces. Patient recovered. He was discharged on Jan. 16.

TABLE VIII.

N. S., White Male Patient, Age Twenty-Two Years, Weight Sixty Kilograms. Diagnosis: Pneumonia, Lobar; Consolidation of Right Lower Lobe; Empyema.

Date.	Day of disease.	Urine in c.c. / Specific gravity at 15° C.	Furfurol-phloroglucid in gm.	Glucuron in gm.	Rectal temperature, C. Maximum. Minimum.	Intake and remarks.
1912						
Dec. 8	10	1,820 / 1,016	0.375	1.025	40.5°	*Drugs:* codein 96 mg.
					39.5°	*Food:* water 2,000 c.c., milk 300 c.c., orange juice 250 c.c., lemon juice 100 c.c., sugar 30 gm.
						White blood cells: 20,000.
Dec. 9	11	1,880 / 1,017	0.435	1.305	39.9°	*Drugs:* codein 32 mg.
					39.0°	*Food:* water 2,000 c.c., lemon juice 170 c.c., albumen 70 c.c., tomato soup 70 c.c.
Dec. 10	12	2,330 / 1,016	0.421	1.263	40.2°	*Drugs:* codein 64 mg.
					39.6°	*Food:* water 2,500 c.c., milk 150 c.c., broth 500 c.c., albumen 50 c.c., orange juice 350 c.c., soup 200 c.c., broth 500 c.c., cocoa 350 c.c.
Dec. 11	13	2,200 / 1,016	0.387	1.161	40.8°	*Drugs:* calomel 64 mg., magnesium sulphate 15 gm., codein 32 mg., morphia 22 mg.
					39.5°	*Food:* water 3,000 c.c., milk 350 c.c., lemon juice 180 c.c., broth 900 c.c., albumen 30 c.c.
Dec. 12	14	1,750 / 1,016	0.321	0.963	40.0°	*Drugs:* calomel 196 mg., morphia 22 mg.
					39.8°	*Food:* water 3,000 c.c., milk 200 c.c., lemon juice 30 c.c., soup 200 c.c., albumen 30 c.c., broth 900 c.c.
Dec. 13	15	2,030 / 1,017	0.374	1.122	40.5°	*Drugs:* morphia 11 mg.
					39.4°	*Food:* water 1,700 c.c., milk 800 c.c., broth 300 c.c., soup 150 c.c., orange juice 300 c.c., lemon juice 20 c.c.
Dec. 14	16	1,770 / 1,016	0.317	0.951	40.2°	*Drugs:* morphia 11 mg., codein 32 mg.
					39.5°	*Food:* water 2,000 c.c., milk 550 c.c., broth 650 c.c., orange juice 100 c.c., lemon juice 15 c.c.
Dec. 15	17	1,510 / 1,016	0.287	0.861	40.3°	*Drugs:* calomel 96 mg., codein 48 mg.
					39.6°	*Food:* water 2,000 c.c., milk 200 c.c., broth 670 c.c., orange juice 190 c.c., albumen 30 c.c.
Dec. 16	18	1,600 / 1,016	.309	0.927	40.3°	*Drugs:* codein 24 mg.
					39.6°	*Food:* water 2,000 c.c., milk 400 c.c., broth 500 c.c., albumen 30 c.c., orange juice 280 c.c., ice cream 60 gm., lemon juice 10 c.c., soup 150 c.c.
Dec. 17	19	1,610 / 1,018	0.352	1.066	40.0°	*Drugs:* none.
					39.0°	*Food:* water 1,700 c.c., milk 600 c.c., broth 550 c.c., albumen 30 c.c., orange juice 200 c.c.
						Patient was operated on a few days later.

TABLE IX.

K. K., White Male Patient, Age Twenty-Three Years, Weight Fifty-Nine Kilograms.

Diagnosis: Bronchopneumonia.

Date.	Day of disease.	Urine in c.c. / Specific gravity at 15° C.	Furfurol-phloroglucid in gm.	Glucuron in gm.	Rectal temperature, C. Maximum. Minimum.	Intake and remarks.
1913						
Jan. 10	12	900 / 1,025	0.254	0.762	39.9°	*Drugs:* codein 24 mg.
					39.0°	*Food:* water 1,300 c.c., milk 800 c.c., orange juice 180 c.c., albumen 30 c.c., cocoa 100 c.c., egg 1.
						White blood cells: 8,000.
Jan. 11	13	900 / 1,029	0.243	0.729	39.5°	*Drugs:* codein 24 mg.
					38.9°	*Food:* water 600 c.c., milk 1,600 c.c., orange juice 300 c.c., cocoa 150 c.c., soup 150 c.c., egg albumen 30 c.c.
Jan. 12	14	520 / 1,030	0.149	0.447	39.2°	*Drugs:* codein 24 mg.
					38.0°	*Food:* water 1,600 c.c., milk 500 c.c., orange juice 300 c.c., soup 200 c.c., cocoa 300 c.c., albumen 30 c.c.
Jan. 13	15	1,430 / 1,028	0.350	1.050	38.0°	*Drugs:* codein 16 mg.
					37.0°	*Food:* water 1,600 c.c., milk 800 c.c., cocoa 480 c.c., orange juice 270 c.c., albumen 20 c.c.
Jan. 22	24	1,120 / 1,023	0.204	0.612	37.0°	*Food:* light diet,—milk, eggs, bread, and toast.
Jan. 23	25	1,110 / 1,020	0.188	0.564	37.5°	*Food:* light diet,—milk, eggs, bread, and toast.
					37.0°	
Jan. 24	26	900 / —	0.168	0.504	37.0°	*Drugs:* codein 96 mg.
					36.8°	*Food:* light diet,—milk, eggs, bread, and toast.
						Patient recovered. He was discharged on Feb. 17.

TABLE X.

O. L., White Male Patient, Age Twenty-Six Years, Weight Sixty-Three Kilograms.

Diagnosis: Influenza; Bronchitis, Acute.

Date.	Day of disease.	Urine in c.c. / Specific gravity at 15° C.	Furfurol-phloroglucid in gm.	Glucuron in gm.	Rectal temperature, C. Maximum. Minimum.	Intake and remarks.
1912						20 hr. specimen.
Dec. 3	4	950 / 1,016	0.176	0.528	39.9° / 38.0°	*Drugs:* codein 32 mg. *Food:* water 1,400 c.c., milk 1,100 c.c., eggs 4, soup 150 c.c., orange juice 15 c.c., toast 5 pieces, rice 30 gm., cereal 40 gm., custard 30 gm.
Dec. 4	5	870 / 1,017	0.142	0.436	38.4° / 37.3°	*Drugs:* calomel 64 mg., magnesium sulphate 15 gm., codein 32 mg. *Food:* water 1,300 c.c., milk 1,200 c.c., orange juice 15 c.c., eggs 2, cereal 60 gm., potatoes 30 gm., custard 60 gm., pears 2, pear juice 40 c.c., toast 3 pieces.
Dec. 5	6	950 / 1,020	0.151	0.453	37.8° / 37.0°	*Drugs:* none. *Food:* water 1,500 c.c., milk 1,300 c.c., orange juice 100 c.c., rice 30 gm., bread and toast 10 pieces, cereal 60 gm., eggs 2, bread pudding 75 gm., parsnips 30 gm.
Dec. 6	7	1,430 / 1,020	0.157	0.571	37.0° / 36.8°	*Drugs:* none. *Food:* water 900 c.c., milk 1,200 c.c., orange 1, crackers 2, tapioca pudding 30 gm., potatoes 30 gm., rice 30 gm., squash 30 gm., custard 45 gm., eggs 4, cereal 60 gm., bread and toast 7 pieces. Patient recovered. He was discharged Dec. 11th.

TABLE XI.

Rabbit I. Previously Fed on Cabbage and Bread. Weight at the Beginning of the Experiment, Two Thousand Grams.

Date.	Weight in gm.	Urine in c.c. / Specific gravity at 15° C.	Furfurol-phloroglucid in gm.	Glucuron in gm.	Rectal temperature, C.	Remarks.
1913 Jan. 16	1,820	215 / 1,014	0.0495	0.1485	39.8°	*Intake:* water 100 c.c.
Jan. 17	1,750	135 / 1,013	0.0223	0.0669	39.2°	*Intake:* water 100 c.c.
Jan. 18	1,600 (dead)	130 / 1,012	0.0318	0.0954	40.2°	10 hour specimen. *Intake:* water 50 c.c.; 0.5 c.c. of pneumococcus broth culture intravenously. Death followed after 10 hrs. Blood contained numerous pneumococci.

TABLE XII.

Rabbit II. Previously Fed on Cabbage and Bread. Weight at the Beginning of the Experiment, Two Thousand Five Hundred Grams.

Date.	Weight in gm.	Urine in c.c. / Specific gravity at 15° C.	Furfurol-phloroglucid in gm.	Glucuron in gm.	Rectal temperature, C.	Remarks.
1913 Jan. 16	2,250	260 / 1,013	0.0447	0.1341	39.0°	*Intake:* water 100 c.c.
Jan. 17	2,120	165 / 1,008	0.0190	0.0570	39.0°	*Intake:* water 100 c.c.
Jan. 18	2,070	74 / —	0.0494	0.1482	40.2°	*Intake:* water 50 c.c.; 0.5 c.c. of pneumococcus broth culture intravenously.
Jan. 19	1,900	140 / 1,021	0.0218	0.0664	41.0°	*Intake:* water 50 c.c.; died 12 hrs. after collection of this specimen. Blood contained numerous pneumococci.

DISCUSSION.

On analyzing the cases of lobar pneumonia here reported, one sees that the amount of glycuronic acid excreted in twenty-four hours during the febrile period is, with but few exceptions (tables IV, VI, and VII), more than 0.8 of a gram. Often it is in excess of one gram, reaching sometimes a value of 1.3 grams. Bearing in mind the fact that a normal person of the same weight as the patients studied usually excretes daily on a moderate mixed diet 0.4 to 0.6

of a gram of glycuronic acid, one is justified in drawing the conclusion that there is an increase in the twenty-four hour output of this acid during the febrile period of pneumonia. The amount of increase varies between 0.2 and 0.9 of a gram. In only two cases out of twelve (tables VI and VII) was it impossible to determine a definite increase during the fever.

The question arises as to whether the increased output is perhaps attributable to some special ingredients of the diet, or to the drugs that have been administered to the patient during this period. Inasmuch as the figures show that a direct proportion between the output of fluid and the amount of glycuronic acid excreted is found only within wide limits, such a mechanical factor cannot be held responsible for the increased values observed.

The diet during the period of increased excretion of glycuronic acid consisted mainly of water, milk, orange juice, and albumen water. Some doubt arose as to whether the orange juice might cause an increased output in the urine of substances yielding furfurol. It may be seen in the protocols, however, that the increased excretion of glycuronic acid and the intake of orange juice do not vary together. Several additional experiments were also made on patients convalescing from lobar pneumonia after the resolution of the exudate, in order to see if the orange juice has a definite effect on the amount of glycuronic acid excreted. They were given 200 to 500 cubic centimeters of orange juice daily. The figures obtained, however, did not vary appreciably during the period of observation (table XIII).

TABLE XIII.

K. G., Colored Boy, Eight Years Old, Weight Thirty Kilograms, Convalescing from Lobar Pneumonia.

Date.	Urine in c.c. / Specific gravity at 15° C.	Furfurol-phloroglucid in gm.	Glucuron in gm.	Intake.
1913 April 3	490 / 1,029	0.135	0.405	Milk, bread, potatoes, and moderate amount of meat.
April 4	510 / 1,031	0.154	0.462	Same diet plus 200 c.c. orange juice.
April 5	520 / 1,030	0.134	0.402	Same diet without orange juice.
April 6	440 / 1,031	0.129	0.387	Same diet plus 300 c.c. orange juice.

Some of the patients were given lactose (tables III and IV). In these cases, also, there is no evidence that lactose causes an increase in the urine of bodies that give rise to furfurol. The excretion of glycuronic acid was not higher than in patients not receiving lactose. It may be mentioned, however, that in one fatal case the patient's urine contained a large amount of substances reducing Fehling's solution. Phloroglucin gave, with the distillate, a reddish brown instead of a black precipitate. It was very abundant,—several grams in the twenty-four hour specimen. The results have been discarded.

The patients on whom the observations were made received as drugs only small amounts of codein (not more than 96 milligrams) and morphia (not over 24 milligrams a day). Occasionally they were given calomel and magnesium sulphate. The cases in which larger amounts of glycuronic acid-combining substances were administered (camphor oil, salicylic acid, turpentine) have not been used. The excretion of glycuronic acid in those cases was still higher (table III). It has been also repeatedly found that the intake of 96 milligrams of codein in twenty-four hours does not provoke a marked increase in the output of substances giving rise to furfurol (table IX). Furthermore, in the case of most of the patients studied, the excretion of glycuronic acid has also been estimated during the period of convalescence. The figures obtained in all the cases were lower than those observed during the active pathological process. They are sometimes high (tables I, II, and III), but this is undoubtedly due to the rich diet, especially to the intake of large quantities of meat. The more the diet during convalescence approaches that of the febrile period, the more evident the differences become (tables III, IV, and IX).

From the analytical data it seems justifiable to conclude that the increase in the excretion of glycuronic acid during the febrile period of lobar pneumonia is not due to outside factors, but that it is associated with the pathological process in the body. No accurate observations have been made concerning the length of time during which there is an increased output of glycuronic acid. Probably in most cases the figures return gradually to normal within a few days after the crisis or lysis.

This phenomenon is not peculiar to lobar pneumonia. Similar

observations have been made in postpneumonic empyema (table VIII) and in bronchopneumonia (table IX). However, in a case of influenzal bronchitis (table X), one day after the subsidence of the fever, the amount of glycuronic acid excreted was normal.

The variation in the output of glycuronic acid in general pneumococcus infections in rabbits was studied (tables XI and XII). The experiments were carried out on fasting animals. As seen in the tables, there is also in these cases a marked increase in the output of substances giving rise to furfurol during the active period of the disease.

SUMMARY AND CONCLUSIONS.

1. It has been found that the amount of glycuronic acid excreted in the urine, estimated by Tollens's furfurol distillation method, is increased during the febrile period of almost all cases of lobar pneumonia in man. Patients weighing from fifty-five to seventy kilograms excrete during the febrile period 0.8 to 1.3 grams of glycuronic acid in twenty-four hours, while the output of a normal individual of the same weight, age, and under similar conditions would not exceed 0.4 to 0.6 of a gram.

2. This increased output is not dependent on outside factors; it is attributable to pathological changes of the organism itself during the disease.

3. Similar observations have been made in postpneumonic empyema and bronchopneumonia.

4. Fasting rabbits, with general pneumococcus infection, have also shown a marked increase in the output of glycuronic acid during the pathological process.

Whether the conjugated substances with the glycuronic acid in the cases studied have the same origin and are qualitatively the same as under normal conditions, or whether we are dealing with different compounds, has not yet been determined.

THE HISTOGENESIS OF BLOOD PLATELETS.*

By WADE H. BROWN, M.D.

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PLATE 9.

The problem of the histogenesis of blood platelets has been attacked from many points of view, but none of the theories advanced seems to be wholly satisfactory. Among the causes which have contributed to this confusion is the failure to recognize the unity in character of all blood platelets, and, until recently, the inability to reproduce the structural and tinctorial characteristics of blood platelets in section as they appear in smears stained by any of the stains of the Romanowsky type. The methods of staining blood platelets in section devised by Wright[1] and by Schridde[2] have obviated the technical difficulties of the problem and have served to emphasize the idea of the unity of blood platelets.

By means of a special method of technique Wright succeeded in staining blood platelets in section as characteristically as in smears, and he was further able to demonstrate the existence of a parent protoplasm with identical structure and staining characteristics in the megakaryocytes of the blood-forming organs. This theory of the origin of blood platelets from the megakaryocyte has been confirmed by Bunting,[3] Ogata,[4] and others. It is especially convincing on account of the ease and clearness with which the essential facts can be demonstrated.

In tracing the development of platelets in embryonic and adult

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[1] Wright, J. H., *Boston Med. and Surg. Jour.*, 1906, cliv, 643; *Jour. Morphol.*, 1910, xxi, 263.

[2] Schridde, H., *Anat. Hefte, 1te Abt.*, 1907, xxxiii, 1.

[3] Bunting, C. H., *Jour. Exper. Med.*, 1909, xi, 541.

[4] Ogata, *Beitr. z. path. Anat. u. z. allg. Path.*, 1912, lii, 192.

tissues, however, Wright refers to only two types of cells in mammals that possess a blue staining protoplasm with purple granules analogous to those of the platelet; namely, the megakaryocyte and their forerunners in embryonic tissues, or, as he calls them, "small megakaryocytes." One is left uncertain, therefore, as to whether other tissue cells possess a protoplasm of like character, or whether this type of protoplasm is restricted to the two cells named. We know from smear preparations that this is not the case with the cells of the circulating blood, as the transitional leucocyte possesses a granular protoplasm quite like that of the platelet, and to a less degree azure granulations are occasionally observed in other mononuclear leucocytes. We might infer, therefore, that such narrow limitations as Wright's work implies could not be placed upon the distribution of protoplasm with the characteristic azure granulation of platelets, and the question naturally arises as to whether cells with such protoplasm might not participate, to some degree, in the formation of blood platelets. It was with these points in mind that an investigation of the distribution of platelet-like protoplasm and the participation of this protoplasm in the formation of blood platelets was undertaken.

The material used in the investigation was taken largely from the rabbit and the guinea pig, although some human tissue was used. The Schridde and the Wright methods of staining were used as the basis of the technique. Both methods, especially that of Wright, permit of considerable elasticity in their use. A brief note of some of the points that I have found of advantage in the application and modification of this technique is of importance.

TECHNIQUE.

Tissues.—Tissues for platelets should be obtained as fresh as possible, but good granular stains of megakaryocytes and other cells can be obtained from tissues not fixed for eight to ten hours after the death of the animal.

Fixation.—The saturated solution of bichloride of mercury in 0.9 per cent. salt solution, recommended by Wright, is probably the best fixative for both the Wright and the Schridde stains.

Almost any other fixing fluid will give good results, except for the finer details. Formalin, methyl alcohol, grain alcohol, Orth's fluid, and even Zenker's fluid preserve both cell granules and platelets and permit of clear, differential stains. Of these, methyl alcohol, Orth's fluid, and formalin can be recommended.

Imbedding and Sectioning.—Any method of paraffin imbedding is permissible; there is little or no choice so far as the effect upon the staining is concerned. Sections should be as thin as possible.

Staining.—This is the important feature of the technique, and with both the Wright and the Schridde stains it must be varied according to the fixation of the tissue and the results desired. The proportion of polychrome methylene-blue to eosin now recommended by Wright is 1 to 10.[5] I have found that it is essential to vary these proportions according to the fixation of the tissue in order to procure the best results, and suggest the following scheme of variations in the composition of the staining mixture with an average preparation of the polychrome methylene-blue, according to Wright, stated in volumes of the eosin solution to one volume of the polychrome methylene-blue: after fixation with bichloride of mercury, 8 to 10 volumes of eosin; after fixation with methyl alcohol, 10 to 12 volumes of eosin; after fixation with formalin or Orth's fluid, 12, 14, or 16 volumes of eosin; after fixation with Zenker's fluid, 7 or 8 volumes of eosin. The mixtures are not stable and should be prepared and diluted with an equal volume of distilled water immediately before use in order to secure constant results. With the Giemsa stain, used by Schridde, similar variations in dilution are advisable. The time of staining varies from ten to thirty minutes, as the proportion of eosin in the mixture is increased. The weaker methylene-blue mixtures with a longer time in a warm oven are preferable. The best results, considering all types of cells, are obtained with tissues fixed in Wright's bichloride of mercury salt solution and stained with Schridde's stain.

Dehydrating, Clearing, and Mounting.—These steps are best carried out with pure acetone, oil of turpentine, and turpentine colophonium, as indicated by Wright.

[5] Mallory, F. B., and Wright, J. H., Pathological Technique, 5th edition, Philadelphia and London, 1911, 373.

The adjustment of the proportions of the components of the staining mixture to the fixation or staining affinity of the tissue is of prime importance. Formalin-fixed tissue is difficult to stain with eosin, while tissue fixed in bichloride of mercury has a strong affinity for this stain, and this principle has been found applicable to the polychrome methylene-blue mixtures. The mixtures of Wright's stain with small amounts of methylene-blue are especially advantageous in demonstrating cell granules, but are apt to stain the cytoplasm of platelets extremely faintly or not at all. A stain that contains sufficient methylene-blue to impart the characteristic blue color to the platelet protoplasm will inevitably overshadow much of the finer granules of other elements, except the megakaryocytes. The staining of these cells is most brilliant with the Wright stain. The superiority of the Giemsa stain with tissues fixed in bichloride of mercury is that all elements are brought out clearly and characteristically.

OBSERVATIONS.

No exhaustive investigation of the occurrence of protoplasm with azure granulations has been attempted, but sufficient work has been done to show that it is not confined to platelets and megakaryocytes nor yet to blood or marrow cells. Such protoplasm has been found in the embryonic trophoblast, the foreign body giant cell with abundant protoplasm, the well preserved tubercle giant cell, the giant cells of sarcomata, and the syncytial masses of chorionic epitheliomata. None of the cells shows a cytoplasm that could be regarded as identical with the cytoplasm of the megakaryocyte, except the syncytial masses of trophoblast and the giant and small cells of the giant-celled osteosarcoma. The other cells mentioned show pale to dark blue cytoplasm and usually dust-like granulations that are easily overshadowed if the cytoplasm be stained too intensely blue. The fragments of such protoplasmic masses are usually abundant in the tissues and strongly resemble platelets. In fact, cell detritus and granular precipitates of many kinds may show the purple granular staining, but the absence of the hyaline blue cytoplasm usually distinguishes such detritus from blood platelets.

It is obvious that such cells and cell detritus are in no way con-

cerned with platelets, and attention is called to them merely to emphasize the fact that the protoplasm is not restricted to cells forming platelets, lest it be assumed that the possession of such protoplasm by cells of the marrow or blood implies a parental relationship to the blood platelet. On the other hand, it should be understood that all megakaryocytes do not necessarily show the same degree of granular staining or, in fact, any granulations at all. The number, size, and staining tone of megakaryocyte granules are extremely variable.

The existence of a group of cells in the circulating blood with platelet-like granulations has been referred to. In the young rabbit these cells are more numerous than in the adult, and a study of the spleen and femur marrow of young rabbits showed that the cells were clearly demonstrable with dilute methylene-blue mixtures of Wright's marrow stain. In order to study these cells with greater advantage, procedures were adopted which destroyed the platelets in the circulating blood and caused proliferation of marrow cells. Injections of alkaline hematin were largely used for this purpose, as its effect upon the various elements of the blood and marrow had recently been worked out, and it gave promise of the type of reaction desired.

In marrow thus stimulated to heightened activity, the endothelial cells covering the surface of the marrow begin to proliferate; they increase enormously in size and become multinucleated, their cytoplasm is vacuolated and stains blue with a rich purple granulation. The granules are irregular; some are extremely fine while others are comparatively coarse. The cell shown in figure 1 is such a cell in an early stage of hyperplasia. These cells continue to increase in size, becoming large multinucleated syncytial masses with pseudopods that burrow into the depths of the marrow. Occasionally one of these pseudopods penetrates the wall of a capillary vessel and segments into small round, oval, or irregular masses with the typical structure and staining of blood platelets. The character and staining of the cytoplasm, the arrangement of granules, and mode of segmentation of the pseudopods of these cells are practically identical with those of the megakaryocyte, except that the processes are usually finer, shorter, and segment more rapidly. The platelets, therefore,

are smaller and more irregular than those formed from the megakaryocyte, but are identical in other respects. Figure 2 shows an endothelial cell mass of this nature, a polykaryocyte, and demonstrates conclusively the impossibility of confusing these structures with the megakaryocytes as well as the fact that they give rise to structures that, from all available criteria, we must regard as blood platelets.

Most of the processes from these hyperplastic endothelial cells are not directly concerned with platelet formation, however, but, as they burrow into the depths of the marrow, segment off from the parent mass and form a new generation of round, oval, or irregular cells. Some of these are distinct myeloblasts with scant dark blue staining cytoplasm and dust-like purple granules that are distinguished with difficulty, while others are larger and possess a more abundant dark blue to pale blue staining cytoplasm with coarse or fine purple granules. These cells are neither ordinary myeloblasts nor myelocytes. In rabbit marrow they are readily distinguished from myelocytes as there are no myelocytes that possess azure granulations. To bring out this distinction, marrow fixed with bichloride of mercury and stained with the Giemsa stain is of especial advantage. In rabbits that have received several large doses of alkaline hematin intravenously the platelets are reduced to a very low level and the megakaryocytes in the marrow of the femur show pyknotic nuclei and a hyaline cytoplasm with few or no granules. The megakaryocytes are necrotic and functionless. The marrow now shows a rapid and peculiar hyperplasia, the cells formed being mainly the large mononuclear or transitional cells with pale blue staining cytoplasm and fine purple granules. Various orders of cells that pass by slight gradations from the ordinary myeloblast to the megakaryocyte can be identified. Some are like the transitional leucocyte of the blood, and the entire group is comparable to the embryonic forerunners of the megakaryocyte.

The cytoplasm of the cells is frequently ragged or shows distinct pseudopods, some of which extend into adjacent capillaries, and the cells themselves enter the blood stream in large numbers. Within the vessels of the marrow most of the cells preserve a comparatively clear cut outline, while only a few show fine, irregular streamers of

platelet-like protoplasm segmenting into platelets. In the splenic sinuses, where they collect in large numbers, the segmentation into platelets is more abundant. Figure 3 shows some of the more common appearances presented by the cells in the splenic sinuses.

Examination of the blood shows an increased number of large mononuclear and transitional leucocytes and an exceptionally large number of these cells with azure granulations. The morphology of the cells in smears is much the same as in sections from the marrow and spleen.

As far as this group of cells is concerned, poisoning with saponin produces the same type of reaction. The myeloid metaplasia occurring in the spleen shows clearly the hyperplasia of premegakaryocytes and the formation of platelets directly from them. This mode of platelet formation has been observed once in the spleen of an old rabbit with a spontaneously developed aplastic condition of the marrow. The case shows that such a mode of platelet formation may exist under pathological conditions that are not of experimental origin.

The morphological evidence from marrow, spleen, and blood points clearly to the formation of blood platelets from the group of marrow and blood cells described above under conditions of excessive demand and incapacity of the megakaryocytes. This evidence was further strengthened by parallel studies of blood and marrow which showed that, after destruction of platelets and megakaryocytes, regeneration of platelets to the normal might occur while there was yet but slight evidence of recovery of the megakaryocytes.

Numerical changes in the elements of the circulating blood furnish still further evidence of a relationship between the mononuclear transitional cells with azure granulations and the blood platelets. The increase in absolute numbers of the cells and the increase in platelets after intravenous injection of hematin are practically parallel. As the normal platelet count is reëstablished the cells gradually sink to their normal level. It is recognized that such changes as these may be only a matter of coincidence, and as the majority of blood platelets are probably formed in the marrow or spleen, under all conditions, evidence of this character is only suggestive. Still, there is at least one condition, Hodgkin's disease, in

which the high platelet and transitional leucocyte counts occur together as the most characteristic features of the blood picture.[6]

DISCUSSION.

My investigations have confirmed much of the work of Wright, but they indicate that some modification or extension of his theory of the origin of blood platelets is necessary. The evidence of formation of blood platelets from the megakaryocyte is conclusive. Except for the origin of platelets in the early embryo from a forerunner of the megakaryocyte, Wright recognizes no other source for blood platelets in mammals. Although the megakaryocyte is undoubtedly the principal source of blood platelets in normal adult life, there is sufficient evidence to indicate that the embryonic forerunner, or homologue, of the megakaryocyte, while progressively decreasing with age, never entirely disappears from either the blood-forming organs or the circulating blood, in the latter situation being represented by the transitional leucocyte. It is uncertain to what degree these cells participate in platelet production under normal circumstances. Although I believe that some platelets are normally derived from transitional leucocytes, the evidence to support this belief comes largely from the embryological relations of the cells and their unmistakable participation in platelet formation under pathological conditions. The evidence, therefore, is not conclusive.

Under experimental conditions it has been found possible to destroy the platelets in the circulating blood in large numbers and, at the same time, partially or completely to incapacitate the megakaryocytes. In such instances the regeneration of platelets from giant endothelial cells in the marrow and from premegakaryocytes and transitional cells in the marrow, spleen, and blood is sufficient to disprove the idea of the exclusive origin of blood platelets from the megakaryocyte, unless we accept for all of these cells the designation of small megakaryocytes, applied by Wright to the forerunners of the megakaryocyte in the embryo. Under conditions of such excessive demand for platelets as cannot be met by the normal mechanism of platelet production, there is undoubtedly a greater

[6] Bunting, C. H., *Bull. Johns Hopkins Hosp.*, 1911, xxii, 369.

or less reversion to an embryonic mode of formation in which cells of a less highly specialized variety assume the rôle of platelet production.

SUMMARY.

1. Under normal circumstances blood platelets are largely derived from the megakaryocyte of the blood-forming organs.

2. The transitional leucocyte, representing a persistent form of the embryonic premegakaryocyte, is a circulating homologue of the megakaryocyte and probably plays some part in normal platelet formation.

3. Under conditions of excessive demand for platelet production, there may be a greater or less reversion to an embryonic mode of platelet formation in which less highly specialized cells than the megakaryocyte participate in platelet production.

4. In addition to the megakaryocyte, the cells that have been observed to take part in platelet formation are hyperplastic endothelial cells in the marrow, and mononuclear and transitional cells (premegakaryocytes) in the marrow, spleen, and blood.

EXPLANATION OF PLATE 9.

The water color drawings were all made with the aid of a camera lucida, and with a Leitz No. 4 ocular and 2 mm. objective. All the figures are from formalin-fixed tissue stained by Wright's method, except the central cell in figure 3, which is from tissue fixed in bichloride of mercury and stained with the Giemsa stain. The composition of the Wright stain used in figures 1 and 2 was methylene-blue 1 part, eosin 12 parts; in figure 3, methylene-blue 1 part, eosin 14 parts.

FIG. 1. An endothelial cell from the surface of the femur marrow of the rabbit, illustrating an early stage of hyperplasia.

FIG. 2. A cell of the same type and situation as that in figure 1, showing marked hyperplasia. A stubby mass of protoplasm in the center is projecting into a capillary and breaking up into blood platelets. The more prolonged mass to the right shows no segmentation, while the process to the left is burrowing through the connective tissue covering the marrow.

FIG. 3. Three types of cells that were numerous in the splenic sinuses of one of the rabbits injected with hematin. The presence of various forms of pseudopods and segmentation into platelets is shown. The platelet count in this animal rose to normal, while the megakaryocytes had regenerated to a very slight degree.

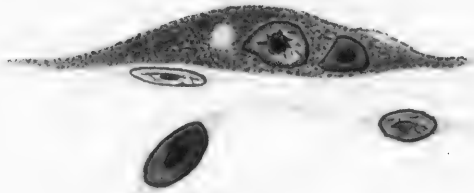

FIG. 1

FIG. 2

FIG. 3

(Brown: Histogenesis of Blood Platelets.)

CONTRIBUTIONS TO THE STUDY OF THE MECHANISM OF THE GROWTH OF CONNECTIVE TISSUE.*

By ALEXIS CARREL, M.D.

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PLATES 10 TO 18.

In previous articles[1] it has been shown that connective tissue cells can be preserved permanently *in vitro* in a condition of active life. Strains of these cells have now been proliferating rapidly outside of the organism for more than sixteen months. In the course of experiment it was observed that a constant relation existed between the rate of growth and the composition of the medium. This fact indicated that certain cell phenomena of the higher animals, such as multiplication, growth, and senility, might now be investigated profitably. Since the time of Claude Bernard it has been known that the life of an organism is the result of the interactions of the cells of which it is composed and of their *milieu intérieur*. But the nature of the interactions has not yet been ascertained; for in order to discover the laws by which they are regulated it would be necessary to modify the humors of the organism and to study the effect of these modifications on the growth of the tissues. This could not be done on account of the lack of a proper method; but this investigation is now rendered possible because of a technique which permits strains of connective tissue cells to multiply indefinitely *in vitro*, like microörganisms.

I have attempted to ascertain some of the relations which exist between the tissues and their medium, by examining the way in which the medium reacts upon the dynamic condition of the cells

* Received for publication, June 4, 1913.

[1] Carrel, A., *Jour. Exper. Med.*, 1912, xv, 516; Pozzi, F., *Bull. de l'Acad. de méd.*, 1912, lxvii, 475; Ebeling, A. H., *Jour. Exper. Med.*, 1913, xvii, 273.

of a tissue, and how, in turn, the cells modify the properties of the medium. Nearly all the experiments were carried on during the first months of 1913 with strains of connective tissue cells derived from tissues extirpated from embryonic or adult chickens during the months of January and June, 1912, and February, 1913. The fragments of tissue composed of these cells were preserved in a medium composed of two parts of adult chicken plasma and one part of juice taken from a chick embryo eight days old. Every two or three days the tissue was removed from the culture medium, washed for thirty seconds or one minute in Ringer solution, divided into two or three parts if its volume had increased, and placed in new medium at a temperature of 39° C. This fragment soon surrounded itself with a large number of cells, which formed a new tissue. After forty-eight hours the ring of new tissue was measured with a micrometer. Its width was usually 76.5 or 91.8 microns. The rate of growth remained constant as long as the composition of the culture medium was not modified.

THE INFLUENCE OF THE MEDIUM ON THE ACTIVITY OF THE CELLS.

The effect of the medium upon the rate of proliferation of the cells was studied in two series of experiments. In the first series fragments of tissue composed of cells of known activity were placed in media of different composition. In the second series strains of cells of varied conditions of activity were placed in media of the same composition. By measuring the growth of the fragments of tissue which had been living under these different conditions for several generations it was possible to ascertain in what manner the media affected the development of the tissue.

Series 1.—In the first experiments the influence of different media was studied. Normal adult chicken plasma was the basis of the medium but it was changed in many different ways by increasing or diminishing its osmotic tension, by diluting it, by modifying its reaction, and by adding certain organic or inorganic substances to it. The rate of growth of a given strain of cells cultivated in the different media varied according to their composition. The effect of a given medium was ascertained only when the tissues had been developing in it for several generations.

The influence on tissues of normal adult chicken plasma was first studied. It was found that the rate of development of a tissue cultivated in plasma alone varied according to certain conditions of the animals from which the plasma was taken. The plasma of some chickens was more activating than that of others, and the greatest difference in the results was caused by the varying ages of the animals,—the younger the animal that supplied the plasma the greater the growth of the tissue. Quantitative experiments were made by employing plasma taken from chickens aged four and five months and aged five and six years. It was found that after three days the size of the ring of new tissue, in the plasma of an old animal, was 30.6 to 38.25 microns, whereas in the plasma of a young animal, it was 45.9 or 53.55 microns. When the plasma of an adult animal, whether chicken or dog, was used, the connective tissue lived for several weeks, but the mass of tissue did not increase. In the first experiments, which were performed in October, 1911, in connection with the rejuvenation of cultures, the fragments of tissue were cultivated in the plasma of an adult animal.[2] The increase in volume was so slight that the loss of cells caused by the section of the medium and the passages deprived the culture of nearly all the new tissue formed during its period of active life. After fifty-five days the fragments of connective tissue which had been growing constantly were no greater than on the first day. This proved conclusively that the plasma of an adult animal did not possess the power of producing a large increase of the mass of the tissue. It was next attempted, by modifying the conditions of the medium, to impart to the plasma of an adult animal a dynamic power analogous or superior to that possessed by the plasma of a young animal. It was found that in a medium composed of plasma and of embryonic juice the volume of tissue increased with great rapidity. The tissue grew so much that it became necessary to divide and subdivide it. Thus a large number of new cultures were made from tissues derived from the few fragments extirpated in January and June, 1912. This activating power is not peculiar to embryonic juice, for extracts taken from

[2] Carrel, A., *Jour. Am. Med. Assn.*, 1911, lvii, 1611.

most of the organs of an adult animal possess this property in a lesser degree. When an animal is old, the juice becomes less activating. Whereas the muscle extract of a young chicken produced in two days a ring of new tissue 76.5 or 91.8 microns in width, that of an old fowl produced a ring of only 22.95 microns.

The action of media of different composition on a given strain of cells was studied in a great many experiments. A fragment of tissue composed of cells which had been living for several months *in vitro* was divided into two equal parts. One part was cultivated in normal plasma and the other in plasma containing different quantities of embryonic juice or other substances. The difference in the quantity of tissue produced in forty-eight hours around the fragments cultivated in plasma alone and mixed with embryonic juice was very large (figures 1 and 2). After several passages the growth of the tissue living in normal plasma became progressively reduced and often stopped completely. On the other hand, the growth of the tissue cultivated in a mixture of plasma and embryonic juice was so abundant that it had to be divided repeatedly. This is shown by experiments of which the following is a type.

The tissue contained in culture 5,142, which had undergone 150 passages and which was growing rapidly and regularly, was divided into two parts, A_1 and B_1. Part A_1 was placed in adult chicken plasma, while part B_1 was cultivated in a medium composed of two parts of plasma and one part of embryonic juice. After forty-eight hours the width of the ring of new tissue formed around A_1 was 30.6 microns, while around B_1 it was 91.8 microns (figure 3). A second time A_2 was cultivated in normal plasma and B_2 in plasma and embryonic juice. After forty-eight hours B_2 was surrounded by a ring 91.8 microns in width, while not a single new cell could be seen around A_2 (figure 4). Nevertheless, the tissue of A_2 was not dead, and it started to grow when it was replaced in a mixture of plasma and embryonic juice (figures 5 and 6). By increasing or diminishing the proportion of embryonic juice contained in normal plasma it became possible to regulate the quantity of new tissue produced in forty-eight hours by a group of connective tissue cells. After a few passages a strain of cells cultivated in adult chicken plasma containing a given quantity of embryonic

juice was growing at a constant rate. After more than sixteen months the strains of connective tissue cells were proliferating at the same rate as the original piece of embryonic tissue from which they were taken. Thus it was demonstrated that connective tissue which had been living *in vitro* for more than one year could greatly increase its mass in a short time. A fragment of tissue composed of the strain of cells of January, 1912, which had undergone 130 passages, was photographed (figure 7), then divided, and photographed again (figure 8) after forty-eight hours. These photographs show that the volume of the tissue had at least doubled during these two days. In the experiments performed with the strain of June, 1912, which had undergone eighty-seven passages, the increase in volume was almost identical. A fragment of tissue composed of these cells was photographed (figure 9), and after being divided was transferred to a new medium. Then infection occurred and the bacterial colony was extirpated together with about one fourth of the tissue; but the remaining part proliferated actively and the tissue was divided and subdivided. Other photographs (figures 10, 11, and 12) were taken during the following days. By comparing the first photograph and the photographs taken on the fourth, sixth, and tenth days it is possible to see what enormous quantities of cells were produced from the small original fragments. In ten days the volume of the tissue had increased at least fifteen times. In other experiments fragments of tissue derived from the strain of cells which had lived for more than fourteen months outside of the organism were cut in smaller pieces and suspended in Ringer solution. A drop of the suspension mixed with normal plasma and embryonic juice was placed in various test-tubes. After two days a sphere composed of elongated connective tissue cells had developed around each small fragment (figure 13). When two spheres were sufficiently close they became united by a bridge composed of chains of active tissue cells (figure 13). The increase of the mass was very large, and in one experiment the volume of the tissue augmented more than fifty times in six days. The experiments show the importance that is attached to the composition of the culture medium, since the growth of a strain of cells, the volume of which increased so rapidly in a

proper medium, remained stationary when it was merely cultivated in normal adult chicken plasma. As the rapidity of growth of a tissue depends so much on the composition of the medium, it may become possible to use as a reagent of the dynamic value of the humors of an organism a strain of cells adapted to life *in vitro*. If human connective tissue cells could be preserved in a condition of permanent life, as connective tissue cells of the chicken are preserved, the value of the plasma of an individual might be appreciated by the cultivation in it of a group of these cells and by the observation of the rate of their multiplication. These observations would perhaps give some indication of certain values of the blood of an organism and possibly some clew to its age.

Series 2.—In the second series of experiments, strains of cells of varied activity were cultivated in identical media. Modifications in the rate of growth occurred and the progressive changes of the dynamic condition of the cells under the influence of the medium could be studied. It is known that the dynamic state of different tissues taken from a normal animal, or that of tissues previously cultivated *in vitro* under different conditions, varies largely. Fragments of connective tissue taken from an embryo, or from young adult and old animals and placed in normal adult chicken plasma grew at different rates. The velocity of the growth always varied in inverse ratio to the age of the animal from which the tissue had been extirpated. The various tissues of one animal, or the different parts of one tissue, are not necessarily identical as regards their dynamic condition. In one case, for instance, a small clot of blood in the subcutaneous connective tissue of an adult chicken had become encysted by connective tissue. A few fragments of the cyst wall were cultivated by the ordinary method. After twenty-four hours these fragments of connective tissue were surrounded by a large and dense area of small round and fusiform cells. During the same period of time normal connective tissue from the same region of the body produced only a few large and elongated connective tissue cells, which constituted a network. Two photographs (figures 14 and 15) show the differences in the morphology of slowly and actively growing cells and in the extent of growth after forty-eight hours. The dynamic condition of the connective tissue living

in vitro varied according to the manner in which the cultures were prepared and kept. The greatest activity was observed in the old strain of cells which had been undergoing daily passages for some time past. A comparative study of the proliferation of the cells and of the connective tissue cells of an eight day old chick embryo showed that the quantity of tissue produced in forty-eight hours by the strain adapted to life *in vitro* could be twenty times more abundant than that produced by fresh embryonic tissue. When the tissues were allowed to remain in the medium for two, three, or four days without change, the rate of proliferation decreased. When a fragment of tissue was kept in its medium at a low temperature for four, five, or six days, the rate of growth and the morphological appearance of the cells were modified (figure 16). The cells became larger and their cytoplasm was darker and more abundant. The rate of proliferation had diminished and even after a few repeated passages the growth of the tissue was still slow.

An attempt was then made to determine whether the dynamic condition of the cells was a fixed character that persisted for a long time, or whether it was merely a temporary effect, produced entirely by the medium. Fragments of tissue or strains of cells of different dynamic conditions were placed in identical media, composed either of normal adult chicken plasma or of plasma containing more or less tissue juice. The rate of growth of these tissues which were originally in different conditions of activity was at first very unequal; but after a few passages it became uniform. The strains of cells which had been artificially activated through daily passages, and which in forty-eight hours produced a ring of new tissue of 153.0 or 183.6 microns in width, were, after a few passages, producing a ring of 76.5 or 91.8 microns. Again, the fragments of tissue which originally surrounded themselves in forty-eight hours with a ring of 15.3 or 30.6 microns produced after a few passages a ring of about 91.8 microns. The influence of the medium on the rate of growth was not immediate. For a few days the rate of proliferation depended in large measure upon the previous dynamic condition of the cells; then their activity increased or diminished according to the medium and finally became constant. For instance, a fragment of tissue composed of a strain of cells

which had undergone more than 150 passages, and the growth of which had completely stopped, after having been left for several days in normal chicken plasma, was cultivated in a mixture composed of two parts of plasma and one part of embryonic juice. After twenty-four hours a few cells were seen in the culture medium around the original fragment. After three days the fragment was surrounded by a thin ring, the width of which was 45.9 microns. The fragment was then washed and placed in a medium of identical composition. Two days afterwards the ring of new tissue which had formed was 76.5 microns. After one more passage the rate of growth was entirely normal. The tissues which grew actively and which were placed in the plasma of adult or old animals continued for a few days to develop rapidly. Later the rate of growth diminished progressively. Therefore it seems that the dynamic condition of the cells results from certain substances supplied directly or indirectly by their medium. The activity manifested by the cells for a few days after their medium has been changed may be partly due to the reserves which they have previously accumulated. The action of the medium on the cells is not immediate, but necessarily occurs sooner or later. Hence the conclusion that the dynamic condition of a strain of cells is determined by the medium either immediately or after a short time; or in other words, it is a function of the medium in which it is cultivated.

THE INFLUENCE OF THE CELLS ON THE MEDIUM.

It might be assumed that cells which were actively developing in a medium markedly modified the latter, and rendered it progressively unsuited to their life. Normal death of cultivated tissues was considered as possibly brought about by the exhaustion of the nutritive substances contained in the medium and by the accumulation therein of certain catabolic substances. Some modifications of the culture medium were observed to occur when connective tissue was made to grow actively in Gabritschewski boxes or in test-tubes. The plasmatic jelly around the groups of cells which were rapidly proliferating became thinner, and serum was exuded. The atmosphere of the box became rarefied. When, in cases where tissues were

cultivated in large tubes, the velocity of growth was great, the fibrin which is the framework of the film covering the glass wall soon disappeared and the culture medium appeared to liquefy. The modifications of the atmosphere of the boxes and of the culture medium were merely the manifestations of more profound physico-chemical changes, which will be studied later in greater detail. The only thing which was studied in the present connection was the action on a strain of cells of an extract taken from a culture medium in which normal embryonic tissue had been actively developing. The experiments consisted in adding the fluids from an old culture to the plasma in which the connective tissue cells were being cultivated. The fluids were obtained in the following manner: Normal embryonic tissue was cut into very small pieces, suspended in Ringer solution, and mixed in test-tubes with normal chicken plasma. In some experiments the plasma was allowed to coagulate at the bottom of the tubes; in others, the plasma was made to coagulate in a thin film on the glass wall, whereupon the growth of the tissue became much more rapid. Some of the tubes were placed in cold storage, and the others in the incubator. In the former no growth occurred, while in the latter the tissue grew more or less actively, according to the manner in which the culture had been prepared. After a few days the contents of the tubes were removed. The clot was cut into small pieces and centrifuged and the supernatant fluid as well as the fluid exuded during the growth of the tissue was used for the experiments, one or two parts of the fluid being mixed with one part of plasma. Extracts of cultures were used which had been kept in the incubator in active life or in the refrigerator in latent life. In these media were cultivated strains of connective tissue cells adapted to life *in vitro*. After forty-eight hours the width of the new tissue was measured with a micrometer. The results varied according to whether the cultures that supplied the extract had been kept in the incubator for a short or a long time.

When the embryonic tissues had been growing for one or two days only, the fluid extracted from their medium activated the growth of connective tissue cells. This activating power exceeded that of the fluid extracted from the cultures preserved in cold

storage. Therefore it appeared that the embryonic tissues living actively for a short time had modified the culture medium and rendered it more suitable to the growth of connective tissue cells.

When the cultures of embryonic tissues were allowed to grow more extensively or for a longer time, the properties of their extracts became modified. In one form of experiment the plasma and the suspension of embryonic tissue were placed in tubes and allowed to remain in the incubator for four or five days; in a second, the plasma was distributed over the wall of the tube in a thin layer, the fragments of embryonic tissue being embedded therein at more or less regular intervals. In the latter case the growth was very rapid and in two days the plasma jelly had almost completely liquefied, while the wall of the tube had become covered with continuous and thin layers of living cells. The fluids extracted from the cultures which had been living actively acquired the property of retarding the growth of connective tissue. In a few experiments these fluids caused the proliferation of the cells to cease almost entirely. The experiments show that embryonic tissue which has been actively growing in a culture medium renders it unsuitable for the life of cells.

The results of the experiments lead one to assume that in a culture of tissue progressive diminution of the tissue's rate of growth and its ultimate death are probably brought about by similar modifications of the medium. It was this supposition which enabled me to prolong indefinitely the life of connective tissue *in vitro*. It was found that by washing the tissues of a culture in Ringer solution and placing them in a new medium the rate of their growth did not diminish and death failed to occur. This result could be obtained in several different ways. After a few days of growth in the medium the fragments of tissue were washed in Ringer solution or placed in a large quantity of fluid plasma, where it was thought that the substances which stopped the growth could easily diffuse. When the tissues were transferred to a new medium every twenty-four or forty-eight hours it was unnecessary to wash them; but when they had been allowed to grow for four or five days in a medium the washing in Ringer solution favored the rapidity of their growth. The influence of washing on the growth of tissue was

studied particularly in connection with a strain of cells which had undergone more than 100 passages. Each fragment was divided into two parts, A and B. Part A was washed for thirty seconds or one minute, while part B was placed directly in the new medium. When the tissues were cultivated in a mixture of plasma and embryonic juice and transferred to a new medium every twenty-four or forty-eight hours, the difference in size between parts A and B was very slight and often did not exist at all. However, the new tissue was generally less abundant and less dense in the culture which had not been washed (figure 17, *a* and *b*). The differences were more marked when the tissues were transferred several times without being washed. When the tissues were cultivated in adult chicken plasma without the addition of embryonic juice the difference in growth produced by the suppression of the washing was much more apparent. In this case the tissues which had not been washed often died after a few passages.

The experiments indicate that the growing tissues modify their medium; but the nature of the modifications has not yet been determined.

CONCLUSIONS.

When connective tissue cells have been cultivated for a certain length of time in a medium which has been repeatedly changed, a definite relation arises between the rate of growth of the cells and the composition of the medium. It is possible, by adding to the culture medium a given quantity of certain substances, such as embryonic juices, to foresee the extent to which a fragment of tissue composed of a given strain of cells will increase in a given time. The rate of growth of a strain of cells can be accelerated or retarded by the addition to the medium of activating or retarding substances.

The dynamic condition of a strain of connective tissue cells, which have been living in a given medium for some time, is not a definitely acquired characteristic, but a temporary state, and is the product or function of the medium in which the cells are living, and is readily modified merely by altering the composition of the medium.

A knowledge of the characteristics of the growth of connective tissue described has led to a new result,—the indefinite proliferation of a strain of connective tissue cells outside of the organism. The

strain of connective tissue originally obtained from a fragment of chick embryo heart, which had been pulsating *in vitro* for 104 days, was still actively alive after sixteen months of independent life, and more than 190 passages. The rate of proliferation of the connective tissue sixteen months old equalled and even exceeded that of fresh connective tissue taken from an eight day old embryo. It appears, therefore, that time has no effect on the tissues isolated from the organism and preserved by means of the technique described above. During the sixteenth month of life *in vitro* the cells increased rapidly in number and were able in a short time to produce a large quantity of new tissue. This fact, therefore, definitely demonstrates that the tissues were not in a state of survival, as was the case in certain earlier experiments, but in a condition of real life, since the cells of which they were composed, like microörganisms, multiplied indefinitely in the culture medium.

EXPLANATION OF PLATES.

Plate 10.

Fig. 1. Culture 5,200–1. March 10, 1913, fourteenth passage of connective tissue. Medium, adult chicken plasma. Photograph taken on March 12, 1913.

Fig. 2. Culture 5,200–2. March 10, 1913, fourteenth passage of connective tissue. Medium, two parts adult chicken plasma, one part embryonic juice. Photograph taken on March 12, 1913.

Plate 11.

Fig. 3. Culture 5,166. March 10, 1913, seventy-two hours after the 151st passage. A_1, cultivated in normal plasma; B_1, cultivated in two parts normal plasma and one part embryonic juice.

Fig. 4. Same tissues. March 12, 1913, forty-eight hours after the 152d passage. A_2, cultivated in normal plasma; B_2, cultivated in two parts normal plasma, one part embryonic juice.

Fig. 5. Same tissues. March 14, 1913, forty-eight hours after the 153d passage. A_3, cultivated in two parts plasma and one part embryonic juice; B_3, cultivated in normal chicken plasma.

Fig. 6. Same tissues. March 16, 1913, forty-eight hours after the 154th passage. A_4, cultivated in two parts plasma and one part embryonic juice; B_4, cultivated in normal chicken plasma.

Plate 12.

Fig. 7. Culture 3,579. Photograph taken on January 18, 1913, forty-eight hours after the 130th passage.

Fig. 8. Same tissue. Photograph taken on January 20, 1913, forty-eight hours after the 131st passage.

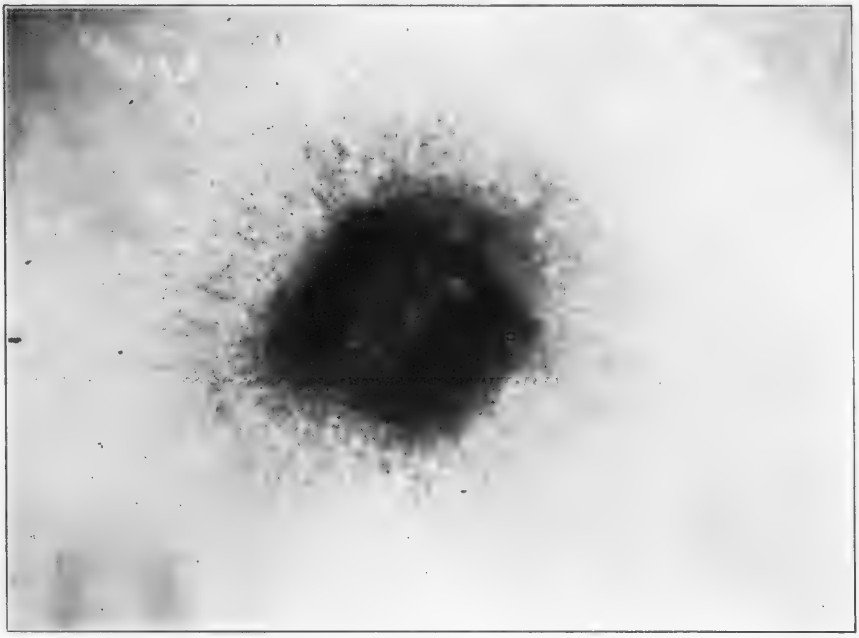


FIG. 1.

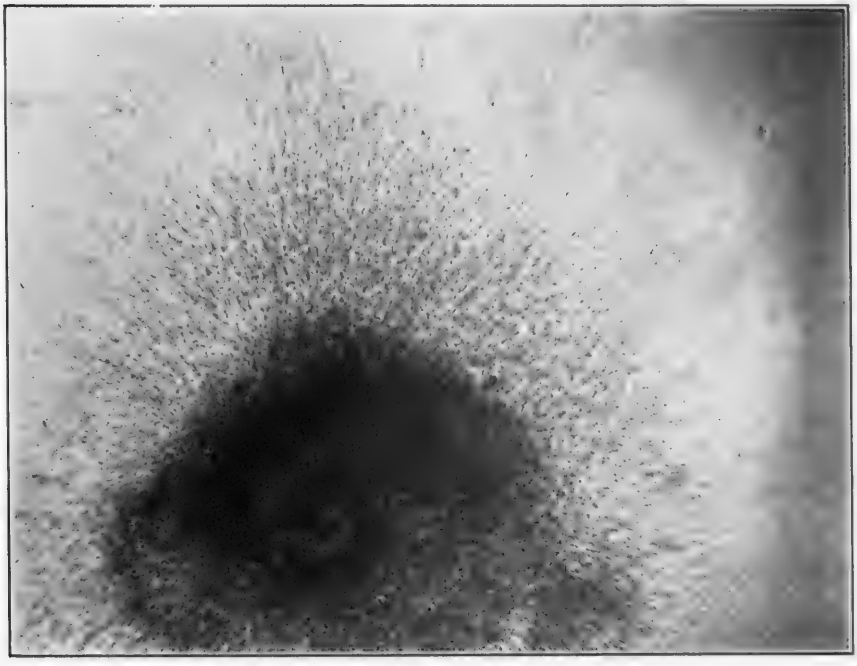


FIG. 2.

(Carrel: Mechanism of Growth of Connective Tissue.)

Fig. 3

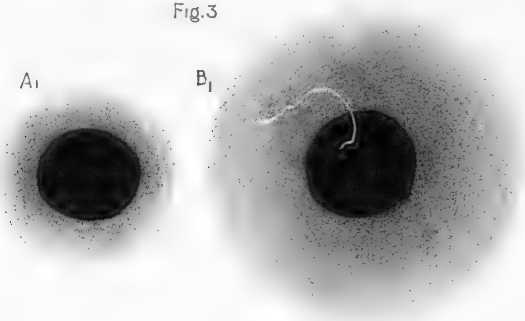


Fig 4

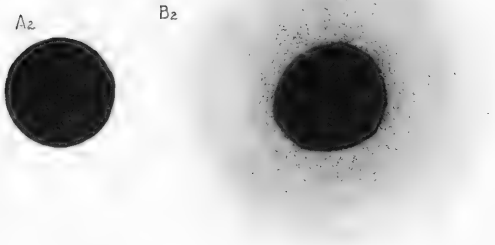


Fig. 5

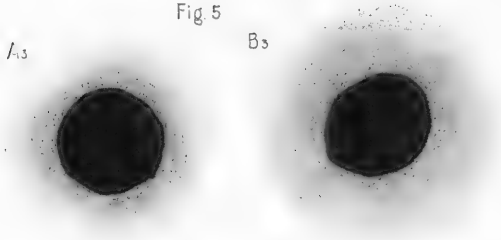
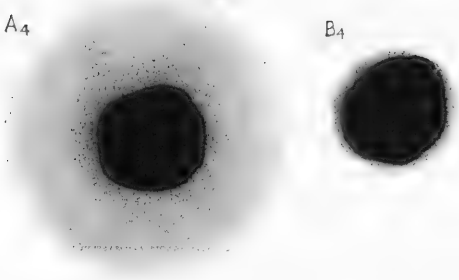


Fig 6



(Carrel: Mechanism of Growth of Connective Tissue.)

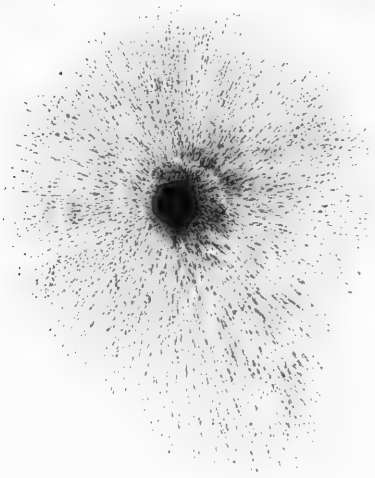


FIG. 7.

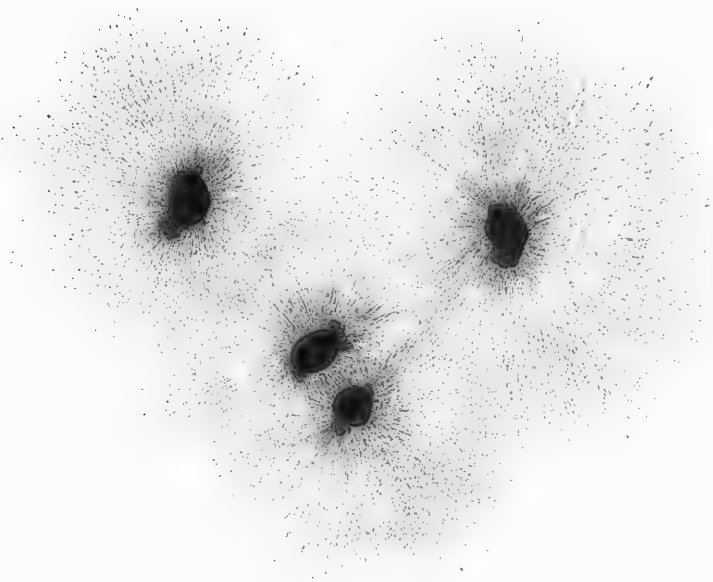


FIG. 8.

(Carrel: Mechanism of Growth of Connective Tissue.)

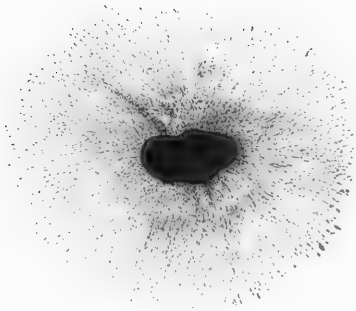


FIG. 9.

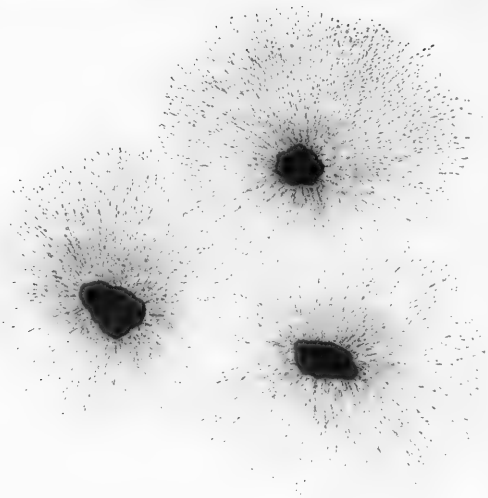


FIG. 10.

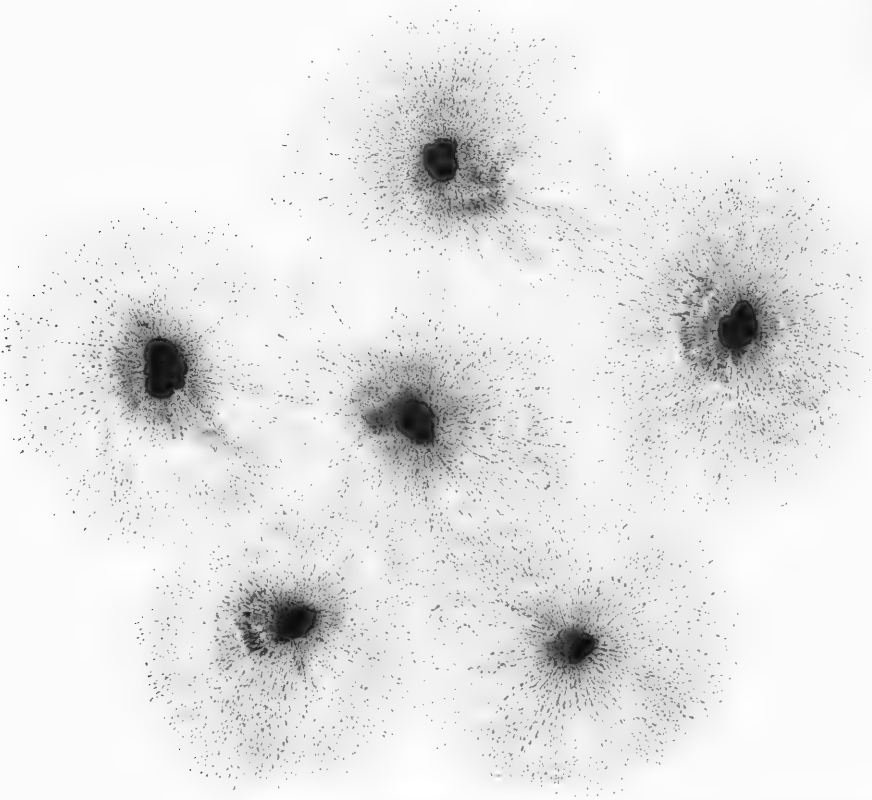


FIG. 11.

(Carrel: Mechanism of Growth of Connective Tissue.)

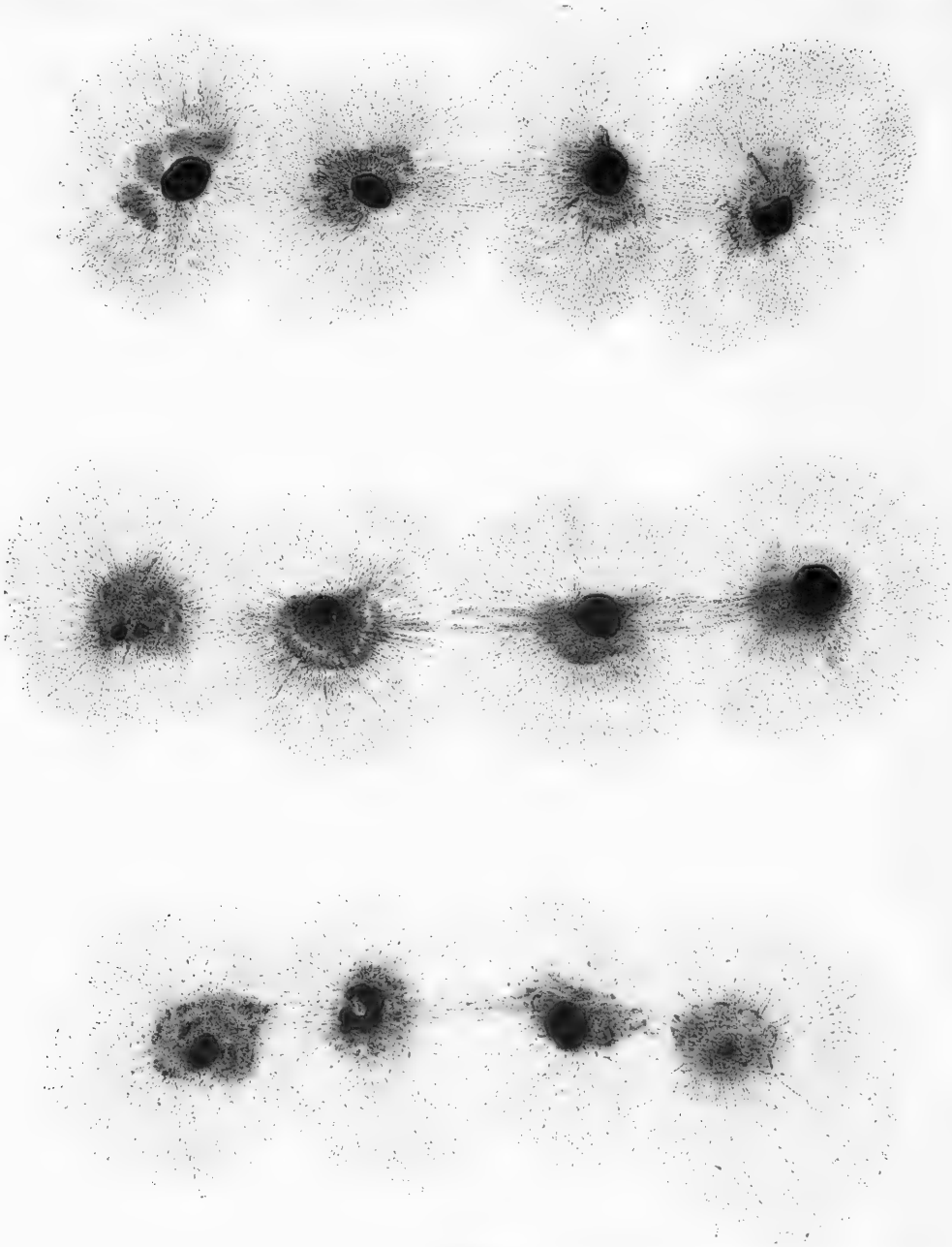


FIG. 12.

(Carrel: Mechanism of Growth of Connective Tissue.)

FIG. 13.

(Carrel: Mechanism of Growth of Connective Tissue.)

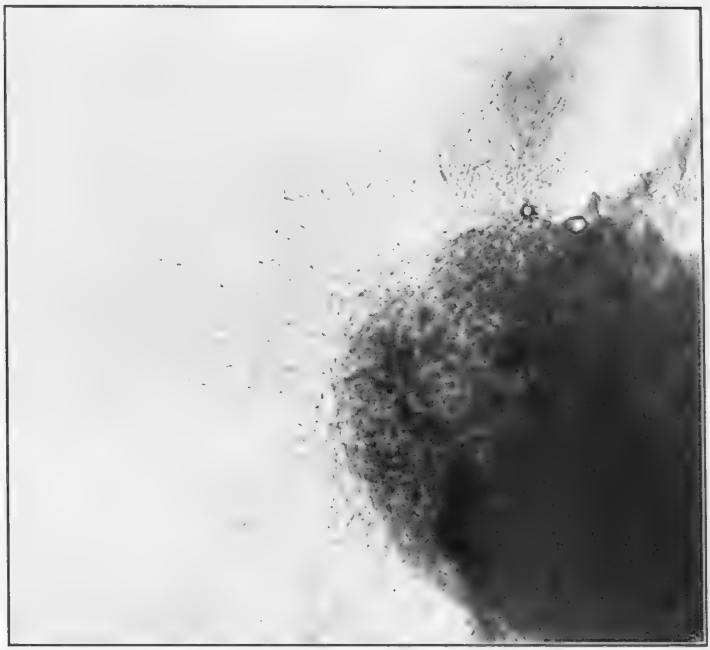


FIG. 14.

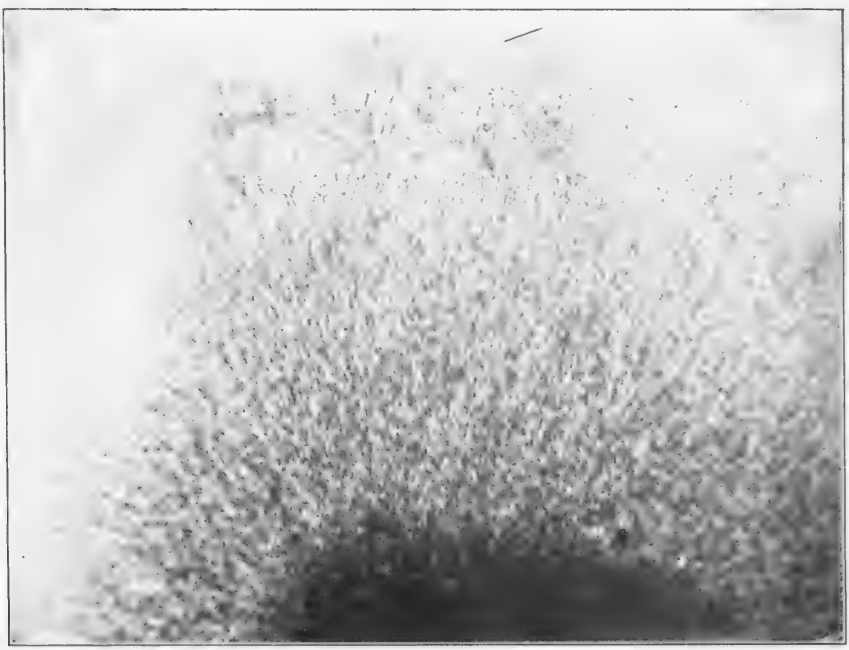
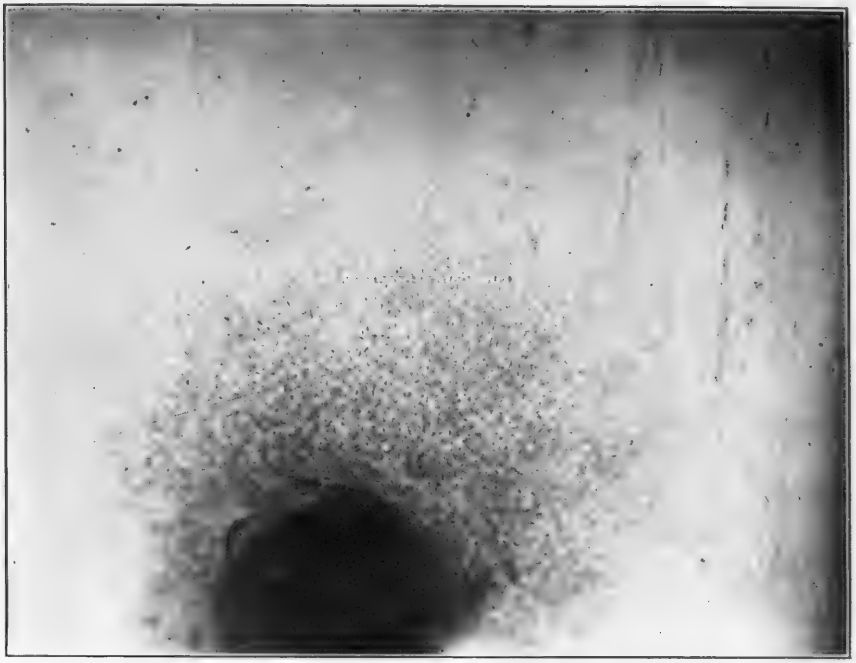


FIG. 15.

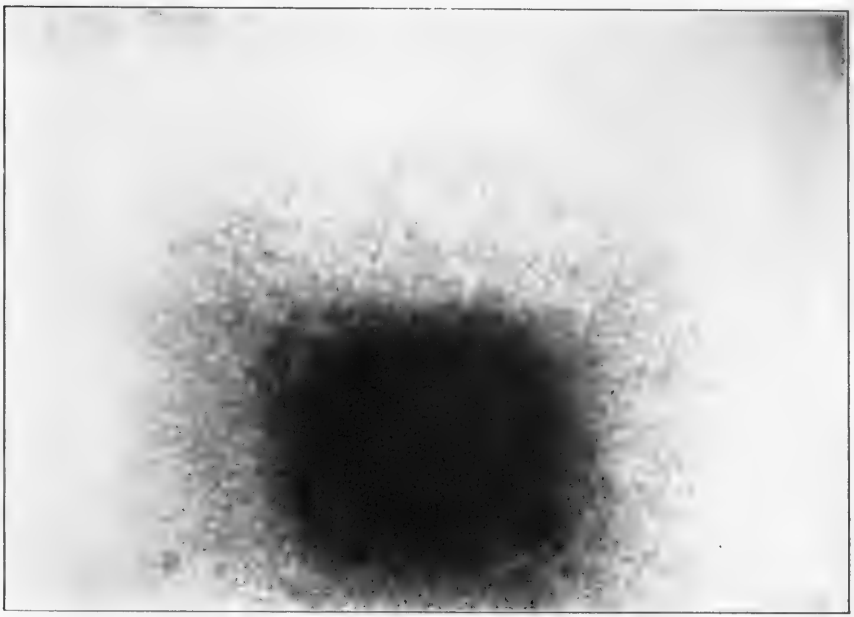
(Carrel: Mechanism of Growth of Connective Tissue.)

FIG. 16.

(Carrel: Mechanism of Growth of Connective Tissue.)



a



b

FIG. 17.

(Carrel: Mechanism of Growth of Connective Tissue.

PLATE 13.

FIG. 9. Culture 3,575. Photograph taken on January 17, 1913, seventy-two hours after the 87th passage.

FIG. 10. Same tissue. Photograph taken on January 20, 1913, forty-eight hours after the 89th passage.

FIG. 11. Same tissue. Photograph taken on January 22, 1913, forty-eight hours after the 90th passage.

PLATE 14.

FIG. 12. Same tissue. Photograph taken on January 27, 1913, seventy-two hours after the 92d passage.

PLATE 15.

FIG. 13. Connective tissue cultivated in a test-tube after fourteen months of life *in vitro*. The photograph shows a growth of forty-eight hours.

PLATE 16.

FIG. 14. Culture of connective tissue of adult chicken, after forty-eight hours.

FIG. 15. Culture 5,200–2. Fourteenth passage of connective tissue, growing very actively.

PLATE 17.

FIG. 16. Culture of connective tissue after seven days in an incubator at a temperature of 35° C.

PLATE 18.

FIG. 17. Culture 5,192. Photograph taken on March 13, 1913, seventy-two hours after the 155th passage. *a* had been washed for one month in Ringer solution. *b* had not been washed.

THE INFLUENCE OF TEMPERATURE ON THE ACTION OF STRYCHNIN IN FROGS.*

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INTRODUCTION.

There is a widespread assumption that in cold blooded animals warming increases the action of all drugs. It is repeatedly pointed out that all chemical, and therefore, presumably, all vital, processes are increased in intensity with rise of temperature, and that the reaction to all drugs is therefore also increased.

Thus Guinard (1) says: "The influence of warmth on therapeutic action is well known. Cl. Bernard stated it long since, pointing out that the sensitiveness of the frog to poisons increases in proportion as the temperature rises. It is a fact which is perfectly logical, because chemical action is the basis of all response to drugs and chemical reactions in general occur more rapidly the higher the temperature. When one administers strychnin to frogs which are each exposed to different temperatures, one notices that rise in temperature accelerates and increases the convulsions." Richet (2) uses similar language. He says: "The influence of temperature on intoxication is particularly marked in cold blooded animals, whose temperature is variable. . . . At a low temperature chemical actions are less intense, and as toxic actions are merely chemical actions, it follows that at a low temperature poisons are less active than at high temperatures. . . . The action of temperature on toxic action is evidently a very general law . . . which may be formulated thus: Toxic actions being chemical actions vary with the temperature of the animal. They are the more active the higher is the temperature of the animal."

In the text-books on pharmacology and physiology there is usually no general statement in regard to the influence of temperature, but in medical journals we meet with the same general view applied to some particular drug. Thus Luchsinger (3), speaking of picrotoxin, refers to Kunde's conclusions, which will be discussed below, and says that summer frogs show much stronger convulsions than winter frogs, and "it seems plausible (*liegt nahe*) to seek in difference of temperature the cause of the great difference in behavior of summer and winter frogs." Schlick (4) says: "Frogs react less to strychnin in the winter, when my experiments were done." The only article, however, which deals directly with the influence of temperature on the action of strychnin in frogs is that of

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Kunde (5), referred to by Luchsinger, which we shall quote at more length. Kunde's experiments were suggested by the fact that frogs react to weaker electric currents when warm, and therefore the cord is less sensitive in the cold and should be less affected by equal amounts of strychnin. He put three frogs in a 1:5,000 solution of strychnin nitrate at 16° C. for ten minutes and then transferred them to dishes containing water at respectively 31° C., 16° C., and 1° C. In ten minutes the frog at 31° C. had tetanus. After fifteen minutes the frog at 16° C. had no tetanus; it was transferred to 31° C. and had tetanus in fifteen minutes. The frog at 1° C. had no tetanus in thirty-five minutes; it was transferred to 31° C. and had tetanus in twelve minutes. The next morning all three appeared normal, but on being put between lumps of ice they developed tetanus, which disappeared when they were warmed, and returned when they were again cooled. On the basis of these experiments Kunde formulated the following law: "With a small dose of strychnin application of warmth stops the tetanus, withdrawal of warmth causes it. With a larger dose warmth brings on tetanus, withdrawal of heat prevents its occurrence." That he considers the effect of temperature on large doses of most importance is plain from his introductory remark. "If a frog is cooled, an electric current which before produced tetanus leaves the animal unaffected. . . . As it has been shown that the cord is also a reagent for strychnin, its effect on the spinal cord must change, if with a constant amount of strychnin present, the temperature of the nerve centers is changed. This hypothesis has, I believe, been confirmed."

From a series of experiments in which we have studied the influence of temperature upon the convulsant action of strychnin, it became evident that the relation of temperature to the tetanic effect of strychnin is erroneously presented when merely expressed in general statements. Neither does an observation of the animal limited to a few hours tell the entire story. In the following we shall report briefly the results which we have obtained in a more detailed study of the influence of temperature upon strychnin tetanus in frogs.

EXPERIMENTAL DATA.

The experiments were performed during the months of January and February on *Rana pipiens*, weighing between fifteen and thirty grams. After injecting in a femoral or the dorsal lymph sac a certain dose of strychnin, the frogs were put in appropriate cages where they were kept at constant, definite temperatures, ranging from 5° C. to 30° C., and observed until recovery took place or several days had passed.

Minimum Convulsant Dose.—In these experiments larger doses, that is, doses which are larger than a reliable minimum, were not

used. Only the effect of minimum doses was studied. But, as will be seen presently, the minimum convulsant dose varies with the temperature at which the animal is kept. A dose which proves to be a reliable minimum at a certain temperature may prove unreliable at another. Only with doses of 0.0006 of a milligram per gram (0.015 to 0.02 of a milligram per frog of twenty-five to thirty grams) did all the frogs show, sooner or later, tetanus at all temperatures. With 0.0005 of a milligram per gram all frogs had tetanus at 5° C., 27° C., and 30° C.; four out of six had tetanus at 13° C., two out of six at 18° C., two out of six at 24° C., and none (of six) at 21° C. With 0.0003 of a milligram per gram all frogs had tetanus at 5° C., while it was inconstant at temperatures above 24° C. and was never seen between 13° and 21° C. With 0.0002 of a milligram per gram three frogs out of five showed tetanus at 5° C., and none at any higher temperature. With smaller doses tetanus was not obtained. That the tetanus occurring at 30° C. is not a heat tetanus is shown by the fact that controls kept at this temperature showed no deviation from normal.

From the above we learn that with a dose of strychnin of 0.0005 of a milligram per gram of frog, only at the low temperature of 5° C. and at the high temperatures of 27° and 30° C. was there a tetanic response in every animal tested, while at various intermediary temperatures the dose failed to be effective in some cases, and at 21° C. failed in every instance. In other words, *a certain dose may be effective at high and low temperatures and be unreliable or fail completely at an intermediary temperature.* This demonstrates the incorrectness of a general statement that strychnin tetanus is favorably or unfavorably influenced by increase of temperature.

We shall reproduce here several abbreviated protocols of experiments with doses of 0.0005 and 0.0003 of a milligram per gram of frog, which may illustrate various points to be brought out in this paper.

In the first experiments a dose of strychnin sulphate equal to 0.0005 of a milligram per gram of frog was injected into the femoral lymph sac.

Frog 102.—Female; weight, 31 gm. Temperature 5° C. January 16, 1913, 9.16 A.M. Injected 0.155 c.c. of strychnin sulphate, 0.01 per cent. 11.30 A.M. Slightly hyperesthetic. 1.00 P.M. On touch the legs fly out in extension and are immediately drawn in. 5.00 P.M. The same. January 17, 9.00 A.M. Animal found in violent continuous tetanus which lasted two days and then gradually passed off. *At 5° C. violent tetanus developed more than 8 hours after injection and lasted for two days.*

Frog 105.—Male; weight, 22 gm. Temperature 5° C. January 16, 1913, 9.54 A.M. Injected 0.11 c.c. of strychnin sulphate, 0.01 per cent. 11.30 A.M. Distinct hyperesthesia. 1.00 P.M. Momentary extension on touch. 5.00 P.M. The same. January 17, 9.00 A.M. In violent continuous tetanus. January 18. The same. January 19. The same. January 20, 9.00 A.M. Still in strong tetanus. 5.00 P.M. Tetanus somewhat less violent. January 21. Markedly spastic but no tetanus. *At 5° C. strong tetanus appeared the day after the injection and continued for about four days.*

Frog 107.—Male; weight, 22 gm. Temperature 13° C. January 16, 1913, 9.22 A.M. Injected 0.11 c.c. of strychnin sulphate, 0.01 per cent. 10.50 A.M. On touch the legs are thrown out stiffly and at once drawn in. 1.00 P.M. Tetanus of brief duration; legs then drawn in. 2.25 P.M. Attacks of longer duration. 5.00 P.M. Strong prolonged attacks of extensor tetanus spontaneously; legs drawn in after attacks. January 17, 9.00 A.M. Almost normal. *At 13° C. tetanus appeared on the day of the injection and was gone the next day.*

Frog 108.—Male; weight, 22 gm. Temperature 13° C. January 16, 1913, 9.56 A.M. Injected 0.11 c.c. of strychnin sulphate, 0.01 per cent. 10.25 A.M. Slightly spastic. 11.05 A.M. Momentary strong extension. 2.00 P.M. The same. 4.00 P.M. The same. 5.00 P.M. The same. January 17, 9.00 A.M. Almost normal. *At 13° C. hardly any tetanus; normal the next day.*

Frog 101.—Male; weight, 25 gm. Temperature 21° C. January 15, 1913, 1.18 P.M. Injected 0.105 c.c. of strychnin sulphate, 0.01 per cent. 1.45 P.M. Slight hyperesthesia. 2.10 P.M. Markedly spastic. 3.00 P.M. The same. 4.00 P.M. The same. January 16, 9.00 A.M. Normal. *At 21° C. no definite tetanus at any time.*

Frog 100.—Male; weight, 20 gm. Temperature 24° C. January 15, 1913, 1.15 P.M. Injected 0.1 c.c. of strychnin sulphate, 0.01 per cent. 1.45 P.M. Slight hyperesthesia. 2.00 P.M. More spastic. 3.40 P.M. Markedly spastic. 5.00 P.M. Slightly spastic. January 16, 9.00 A.M. Normal. *At 24° C. no tetanus at any time.*

Frog 110.—Male; weight, 22 gm. Temperature 27° C. January 16, 1913, 9.36 A.M. Injected 0.11 c.c. of strychnin sulphate, 0.01 per cent. 9.50 A.M. Very lively and active. 10.05 A.M. The same. 10.25 A.M. Sudden strong extensor tetanus; legs not drawn in. 12.00 M. Still in strong tetanus. 1.00 P.M. Less violent. 2.25 P.M. Having attacks of extensor tetanus, but legs drawn in between attacks. 4.00 P.M. Extremely spastic. January 17, 9.00 A.M. Normal. *At 27° C. tetanus in 49 minutes; normal the next day.*

Frog 113.—Female; weight, 30 gm. Temperature 27° C. January 16, 1913, 10.50 A.M. Injected 0.15 c.c. of strychnin sulphate, 0.01 per cent. 11.15 A.M. Spastic. 11.30 A.M. Sudden attack of strong extensor tetanus; legs not drawn in. 4.30 P.M. Becoming less violent. January 17, 9.00 A.M. Almost normal. *At 27° C. tetanus after 40 minutes; normal the next day.*

Frog 128.—Female; weight, 26 gm. Temperature 30° C. January 20, 1913,

11.18 A.M. Injected 0.13 c.c. of strychnin sulphate, 0.01 per cent. 11.45 A.M. Slightly spastic. 11.53 A.M. Extensor tetanus; legs drawn in between attacks. 12.10 P.M. Strong extensor tetanus; legs not drawn in. 12.30 P.M. More violent. 1.00 P.M. Becoming less violent. 3.00 P.M. Recovering. Merely moderately spastic. *At 30° C. tetanus after 35 minutes, recovering the same day.*

In the following experiments strychnin was administered in doses of 0.0003 of a milligram per gram of frog.

Frog 31.—Female; weight, 48 gm. Temperature 5° C. January 7, 1913, 9.41 A.M. Injected 0.48 c.c. of strychnin sulphate, 0.003 per cent. 10.15 A.M. On touch the legs are thrown out stiffly and then drawn in. 4.30 P.M. Condition the same. January 8, 9.00 A.M. In strong continuous extensor tetanus. 2.00 P.M. Still in tetanus. *At 5° C. strong tetanus the next day.*

Frog 144.—Male; weight, 20 gm. Temperature 5° C. January 20, 1913, 5.11 P.M. Injected 0.2 c.c. of strychnin sulphate, 0.003 per cent. January 21, 8.00 A.M. In continuous strong extensor tetanus. 4.00 P.M. The same. 6.00 P.M. Legs drawn in at times. January 22, 8.00 A.M. On touch extensor tetanus lasting several seconds, then legs drawn in. 5.30 P.M. The same. January 23, 9.00 A.M. On touch momentary strong extension. 2.00 P.M. Markedly spastic. 5.00 P.M. Moderately spastic. January 24, 9.00 A.M. Normal. *At 5° C. onset of tetanus not later than 15 hours; lasted several days.*

Frog 74.—Female; weight, 26 gm. Temperature 13° C. January 10, 1913, 9.36 A.M. Injected 0.26 c.c. of strychnin sulphate, 0.003 per cent. 10.00 A.M. Slight hyperesthesia. 11.00 A.M. Slightly spastic. 3.00 P.M. More spastic. 5.00 P.M. The same. January 11, 9.00 A.M. Still spastic. 5.00 P.M. Normal. *At 13° C. no tetanus.*

Frog 82.—Female; weight, 25 gm. Temperature 25° C. January 13, 1913, 11.32 A.M. Injected 0.25 c.c. of strychnin sulphate, 0.003 per cent. 12.05 P.M. Slight hyperesthesia. 12.25 P.M. Markedly spastic. 4.30 P.M. Less spastic. January 14, 9.00 A.M. Normal. *At 25° C. no tetanus.*

Frog 130.—Male; weight, 21 gm. Temperature 30° C. January 20, 1913, 12.13 P.M. Injected 0.21 c.c. of strychnin sulphate, 0.003 per cent. 12.23 P.M. Slight hyperesthesia. 12.30 P.M. The same. 1.00 P.M. In strong continuous extensor tetanus. 1.30 P.M. The same. 3.00 P.M. Legs drawn in between attacks. 4.00 P.M. Markedly spastic; no more tetanus. 4.30 P.M. Slightly spastic. *At 30° C. tetanus after 47 minutes, with full recovery on same day.*

The Length of the Interval between the Injection of Strychnin and the Onset of Tetanus.—The foregoing protocols bring out in the first place the fact already alluded to; namely, that a low temperature as well as a high one may bring on tetanus which may fail to appear at an intermediary temperature. The protocols, however, show also that the length of the interval between the injection and the onset of a definite tetanus seems to be in inverse proportion to the degree of temperature. For instance in experiments 102 and

105, in which the animals were kept at 5° C., a definite tetanus appeared the next day or at least many hours after the injection, while in experiments 110, 113, and 128, in which the animals received the same dose of strychnin (0.0005 per gram of frog), but were kept at 27° C. or 30° C., the interval was less than one hour. Such a relation may be of especial interest, since most of the earlier observations seem to have been based largely upon the early occurrence of tetanic symptoms. This may be the reason why the tetanic effect at low temperatures has been frequently overlooked. In our experiments the animals were kept under observation for several days and the late appearance of tetanic manifestations was scrutinized. Table I shows that while it is true in a general way that high temperatures tend to shorten and low temperatures to lengthen the interval, the figures in the various experiments are too irregular to make them fit into a definite general rule.

Duration of the Tetanic State.—In our experiments notes were taken also regarding the duration of the tetanic state under different temperatures. Here again it may be said, in a general way, that at lower temperatures the duration of the tetanic state is longer than when the animal is kept at higher temperatures. The figures, however, are too variable to press them into a definite rule, as will be seen on examination of the figures in table I. We may note that at 13° C. with doses of 0.0005 and 0.0006 of a milligram per gram tetanus always came on within a few hours and passed off during the following night, while with 0.0003 of a milligram per gram no tetanus was obtained, so that there are no observations on the exact duration of tetanus at this temperature.

Character of the Tetanus.—There is a marked difference in the behavior of the tetanic frog at low temperatures and that at high. In the cold each attack is of long duration, and in the interval between them the animal seems to remain in a certain state of increased tonus. The forelegs are held drawn against the chest, and if the hind legs are flexed passively they extend at once. In a frog having tetanus at a high temperature the attacks are much briefer and in the interval between the attacks there is complete relaxation. When an animal is stimulated soon after an attack has passed off,

TABLE I.

Table Showing for Each Dose and Each Temperature the Number of Frogs Injected, the Number Showing Distinct Tetanus, the Interval between the Injection and the Onset of Tetanus, and the Duration of Tetanus.

Dose 0.0003 mg. per gm.			Dose 0.0005 mg. per gm.			Dose 0.0006 mg. per gm.		
Temperature.	No. of experiments.	Tetanus in.	Temperature.	No. of experiments.	Tetanus in.	Temperature.	No. of experiments.	Tetanus in.
5° C.	11	11	5° C.	10	10	5° C.	10	10
No. of frog.	Before onset.	Duration.	No. of frog.	Before onset.	Duration.	No. of frog.	Before onset.	Duration.
28	144 min.	——	102	7–24 hrs.	——	148	31 min.	——
31	95 min.	——	103	7–24 hrs.	——	149	29 min.	——
32	93 min.	——	104	7–23 hrs.	——	150	34 min.	——
76	8–23 hrs.	——	105	7–23 hrs.	——	151	32 min.	——
77	8–23 hrs.	——	192	7–27 hrs.	——	164	160 min.	——
88	5–21 hrs.	——	193	25 hrs.	——	165	158 min.	——
89	5–21 hrs.	——	222	5 hrs.	80 hrs.	178	66 min.	3 dys.
144	1–15 hrs.	34 hrs.	223	6½ hrs.	73 hrs.	179	94 min.	3 dys.
145	17 hrs.	31 hrs.	224	1–16 hrs.	75 hrs.	190	3 hrs.	3 dys.
146	18 hrs.	24 hrs.	225	1–16 hrs.	80 hrs.	191	7 hrs.	3 dys.
147	1–15 hrs.	40 hrs.						
Average	10 hrs.	32 hrs.	Average	8–16 hrs.	77 hrs.	Average	2 hrs.	3 dys.

Temperature.	No. of experiments.	Tetanus in.	Temperature.	No. of experiments.	Tetanus in.	Temperature.	No. of experiments.	Tetanus in.
13° C.	10	0	13° C.	10	8	13° C.	20	20
			No. of frog.	Before onset.	Duration.	No. of frog.	Before onset.	Duration.
			80	4 hrs.	2–15 hrs.	90	130 min.	——
			98	2¼ hrs.	18 hrs.	91	130 min.	4–22 hrs.
			99	2½ hrs.	4–18 hrs.	92	123 min.	1–15 hrs.
			107	3½ hrs.	4–16 hrs.	93	67 min.	3–20 hrs.
			109	3 hrs.	4–16 hrs.	156	163 min.	4–20 hrs.
			247	3 hrs.	——	157	135 min.	4–20 hrs.
			248	3 hrs.	——	158	194 min.	4–20 hrs.
			249	3 hrs.	——	159	192 min.	4–20 hrs.
						235	136 min.	——
						236	124 min.	——
						237	132 min.	——
						265	22 min.	——
						266	40 min.	——
						267	38 min.	——
						270	89 min.	——
						271	88 min.	——
						286	70 min.	——
						287	88 min.	——
						288	197 min.	——
						289	85 min.	——
			Average	3 hrs.	4–16 hrs.	Average	105 min.	4–15 hrs.

TABLE I.—*Concluded.*

Dose 0.0003 mg. per gm.			Dose 0.0005 mg. per gm.			Dose 0.0006 mg. per gm.		
			Temperature.	No. of experiments.	Tetanus in.	Temperature.	No. of experiments.	Tetanus in.
			18° C.	6	2	16°–18° C.	7	7
			No. of frog.	Before onset.	Duration.	No. of frog.	Before onset.	Duration.
			118	50 min.	2½ hrs.	176	160 min.	——
			119	124 min.	1½ hrs.	177	180 min.	——
						186	85 min.	3 hrs.
						187	148 min.	2 hrs.
						241	54 min.	——
						242	85 min.	——
						243	80 min.	——
			Average	87 min.	2 hrs.	Average	113 min.	2½ hrs.
Temperature.	No. of experiments.	Tetanus in.	Temperature.	No. of experiments.	Tetanus in.	Temperature.	No. of experiments.	Tetanus in.
21°–24° C.	11	2	21°–24° C.	10	2	21°–24° C.	8	8
No. of frog.	Before onset.	Duration.	No. of frog.	Before onset.	Duration.	No. of frog.	Before onset.	Duration.
72	16 min.	6 hrs.	194	120 min.	½ hr.	94	35 min.	4 hrs.
73	14 min.	5½ hrs.	195	96 min.	1 hr.	95	110 min.	5 hrs.
						96	23 min.	2 hrs.
						97	31 min.	2 hrs.
						174	34 min.	3 hrs.
						175	32 min.	4½ hrs.
						212	48 min.	1 hr.
						213	57 min.	3 hrs.
Average	15 min.	5¾ hrs.	Average	108 min.	¾ hr.	Average	46 min.	3 hrs.
Temperature.	No. of experiments.	Tetanus in.	Temperature.	No. of experiments.	Tetanus in.	Temperature.	No. of experiments.	Tetanus in.
27°–30° C.	12	4	27°–30° C.	16	16	27°–30° C.	4	4
No. of frog.	Before onset.	Duration.	No. of frog.	Before onset.	Duration.	No. of frog.	Before onset.	Duration.
78	22 min.	4 hrs.	110	51 min.	5½ hrs.	218	24 min.	3½ hrs.
79	35 min.	4 hrs.	111	57 min.	1¼ hrs.	219	53 min.	3 hrs.
130	45 min.	½ hr.	112	42 min.	3½ hrs.	220	62 min.	2½ hrs.
131	40 min.	2 hrs.	113	40 min.	2½ hrs.	221	60 min.	3½ hrs.
			116	21 min.	1¼ hrs.			
			117	17 min.	——			
			120	25 min.	——			
			121	36 min.	——			
			128	35 min.	2 hrs.			
			129	29 min.	½ hr.			
			138	30 min.	2¼ hrs.			
			139	58 min.	1½ hrs.			
			140	23 min.	1 hr.			
			141	50 min.	½ hr.			
			142	33 min.	2 hrs.			
			143	27 min.	2 hrs.			
Average	35 min.	2½ hrs.	Average	36 min.	2 hrs.	Average	69 min.	3 hrs.

there is no response and the legs if passively flexed are not extended until another attack of tetanus occurs.

DISCUSSION AND SUMMARY.

In order to establish the influence of temperature upon the effect of varying doses of strychnin injected into frogs, the animals must be kept under observation for several days and at various definite degrees of temperature. Statements that the animal was kept "cold," "at room temperature," or "warm" are insufficient. With a certain dose tetanus may result constantly at 30° C. yet never appear at 21° C., and either of these temperatures might be described as warm, when compared to a room temperature of 15° C. Furthermore an animal may apparently fail to respond in the cold to an injection of certain doses of strychnin and yet be found in tetanic convulsions the next day. That an animal may have late, long lasting, or strong tetanus while kept at such a low temperature as 5° C. after an injection of a dose of strychnin smaller than 0.01 of a milligram per frog emphasizes the fact that great caution must be exercised in formulating laws as to the influence of temperature on drug action.

The main results of this investigation may be summarized as follows: Doses of strychnin amounting to 0.0006 of a milligram per gram of frog will cause tetanus at all temperatures between 5° C. and 30° C., although at low temperatures the tetanus may appear late. A dose of 0.0003 of a milligram per gram of frog will frequently produce tetanus at 5° C. as well as at 30° or 27° C., but may nevertheless fail to produce any reaction at such an intermediary temperature as 21° C. Smaller doses, 0.0002 of a milligram per gram, will cause tetanus in the cold but not at high temperatures.

It may be stated in general that in frogs kept at low temperatures the tetanic state sets in later, continues longer, and each tetanic attack is of longer duration, while in the interval between the attacks the state of tonus is higher and the animals are more irritable than when they are kept at higher temperatures.

BIBLIOGRAPHY.

1. Guinard, L., La morphine et l'apomorphine, Paris, 1898.
2. Richet, Ch., La chaleur animale, Paris, 1889, 212.
3. Luchsinger, B., *Arch. f. d. ges. Physiol.*, 1878, xvi, 510.
4. Schlick, K., *Arch. f. d. ges. Physiol.*, 1890, xlvii, 171.
5. Kunde, F., *Virchows Arch. f. path. Anat.*, 1860, xviii, 357.

ON ELIMINATION THROUGH THE MUCOSA OF THE URINARY BLADDER.*

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In a previous communication (1) it was reported that after intravenous injections of large amounts of dextrose into normal rabbits the intestine contained on the average 1.2 per cent. of the injected dextrose, while through the kidneys about 40 per cent. of the injected dextrose was eliminated. In rabbits whose kidneys were removed previous to the injection of the dextrose, and in which, therefore, nearly one half of the injected quantity was prevented from being eliminated, the dextrose content of the intestine reached the average of 2.2 per cent. In discussing these results we arrived at the conclusion that the elimination of 1.2 per cent. of the dextrose in the intestines of normal rabbits can not be looked upon as an "intestinal diabetes" (MacCallum (2)), nor is it due to an increased permeability of the intestinal epithelium (Fischer (3)), nor can the increase of 1 per cent. in the dextrose in the intestine of nephrectomized rabbits be looked upon as a "vicarious action" (MacCallum). It was assumed that the elimination of 1.2 per cent. in the intestine of the normal, and 2.2 per cent. in the intestine of the nephrectomized rabbit after an intravenous injection of a large quantity of dextrose has no specific secretory character, comparable, for instance, with the elimination of dextrose through the kidney. It was suggested that the intestinal elimination is merely due to the temporary hyperglycemia; and, in consequence of the high sugar content of the blood and lymph, elimination occurs in minute quantities through all mucous membranes. In the intestine, on account of the large surface of its mucosa, the sugar eliminated aggregates to the considerable figures of 1.2 and 2.2 per cent.

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The mucosa of the intestine, however, is studded with numerous glands. While each kind of gland secretes a specific substance, all glands eliminate some fluid in which these substances are suspended. The glands derive that fluid from the blood. It is therefore plausible to assume that in taking out some fluid from the blood the glands may be capable of removing from the blood, in very minute quantities and in an unspecific manner, any substance present in the blood in excess of the normal. All mucous membranes are moist; their moisture they also derive from the lymph and the blood. Are mucous membranes as such, without the assistance of glands, also capable of eliminating from the blood substances present in the blood in excess? To test this question, one must examine the contents of a viscus which is lined with a mucous membrane free from glands. Such a hollow viscus is the urinary bladder; its mucosa is not provided with secreting glands. A series of experiments was therefore carried out to determine whether dextrose, when present in excess in the blood, or a substance foreign to the blood, may be eliminated into the bladder. In the following report we shall describe these experiments very briefly.

The rabbit was the experimental animal used. A double nephrectomy was performed and either later on the same day, or on the next day, cannulas were inserted in the bladder and in the jugular vein. Ether anesthesia was used during all operative procedures. In the bladder was inserted a cannula which was modeled on the Ludwig-Spengler cannula for obtaining blood pressure laterally from an artery, except that both plates were made flat. Care was taken, when inserting the cannula, that the circulation was interfered with as little as possible. The cannula was inserted in the ventral side of the bladder and the abdomen sewed up. Warm 20 or 30 per cent. dextrose solution was injected intravenously, seven to ten grams per kilogram being introduced in from twenty to fifty minutes. The bladder was washed by irrigating with warm 0.9 per cent. sodium chloride several times during and after the dextrose injection; the fluid was ejected by the animal as in normal micturition. The washings were boiled immediately, assembled, and concentrated to a small volume on the water bath and analyzed for sugar, qualitatively with Fehling's and Benedict's (4) solutions, and quanti-

TABLE I.

No. of animal.	Color.	Sex.	Weight in gm.	Time of nephrectomy.	Uranin introduced in mg.	Dextrose introduced. C.c.	Per cent.	Gm. per kilo.	Duration of dextrose injection in min.	First bladder washing.	Color of bladder washings.	Uranin found in bladder washings.	Dextrose found in bladder washings.
DB 2	Gray	F.	1,550	Previous day	0	54	20	7	34	After injection of 22 c.c. dextrose solution	Blood-tinged		0.006
DB 3	Gray	M.	2,400	Same day	0	84	20	7	29	After 42 c.c. dextrose solution	Slightly blood-tinged		0.000
DB 5	Gray	M.	2,020	Previous day	0	70	20	7	20	After 57 c.c. dextrose solution	Colorless		0.000(?)
DB 6	Gray	F.	2,030	Previous day	0	70	20	7	33	After 35 c.c. dextrose solution	Almost colorless		0.007
DB 7	Maltese and white	F.	1,440	Same day	0	50	20[1]	7	20	After 25 c.c. dextrose solution	Almost colorless to slightly blood-tinged		0.008
DB 11	Gray and white	M.	1,770	Same day[2]	0	62	20[3]	7	26	After 31 c.c. dextrose solution	Almost colorless		0.000
DB 12	Gray	M.	2,055	Previous day	0	72	20	7	34	1 hour after end of dextrose and thereafter for 1 hour	Colorless to slightly blood-tinged		0.000
DB 13	Maltese and gray	F.	1,420	Previous day	0	50	20[4]	7	31	After 25 c.c. dextrose solution	First very bloody, others almost colorless		0.000
DB 14	Gray	F.	1,740	Same day	20	58	30	10	48	After 5.5 c.c. dextrose solution	Almost colorless	0	0.000(?)
DB 15	Gray	F.	2,140	Same day	100	72.5	30	10	40	After 18 c.c. dextrose solution	Almost colorless to bloody	Trace(?)	0.000(?)
DB 17	Gray	M.	1,625	Same day	100	55	30	10	38	After 11 c.c. dextrose solution	Colorless	0	0.000
DB 18	Gray	M.	1,470	Same day	100	52	30	10	35	After 13 c.c. dextrose solution	Colorless to fluorescent	<0.035 mg.	0.011
DB 19	Gray	M.	1,550	Same day	100	52	30	10	40	After 13.5 c.c. dextrose solution	Colorless to fluorescent	<0.15 mg.	0.000

[1] 50 c.c. $\frac{M}{8}$ magnesium chloride first injected in 21 minutes. Artificial respiration after 18 c.c. had been introduced.

[2] Only ureters tied.

[3] 150 c.c. 0.9 per cent. sodium chloride first injected in 38 minutes.

[4] 50 c.c. $\frac{M}{8}$ calcium chloride first injected in 34 minutes.

tatively by the Pavy-Ramsden method (5). In some of the experiments uranin, the sodium salt of fluorescëin, in very diffusible dye, was dissolved in the injection fluid. This was estimated in the washings by a rough colorimetric analysis.

The results of all the experiments are given in table I. It will be seen that in nine experiments either no sugar or else indeterminable traces were found, and in four experiments six to eleven milligrams, *i. e.*, 0.05 to 0.08 per cent. of the amount injected, were obtained. In three experiments the injection of magnesium chloride, sodium chloride, and calcium chloride, respectively, in physiological concentration, had no noticeable effect upon the result. In the experiments in which twenty milligrams of uranin were introduced, no trace of the dye could be detected in the washings of the bladder. In four experiments 100 milligrams of uranin were introduced; in two there was not a trace and in the other two there was a barely perceptible trace.

In considering these results stress is to be laid on the following facts: (1) the amount of sugar (and uranin) injected was exceedingly great; (2) the kidneys were not functioning; and (3) the bladder was washed at frequent intervals. Nevertheless either there was no dextrose or uranin discovered in the washings or the amounts discovered were quite insignificant.

The conclusion may therefore be drawn that the bladder is practically impermeable for diffusible substances present in the blood in great excess even in the absence of the kidneys, the chief organs of elimination of the body.

BIBLIOGRAPHY.

1. Kleiner, I. S., *Jour. Exper. Med.*, 1911, xiv, 274.
2. MacCallum, J. B., *University of California Publications in Physiology*, 1902–04, i, 125.
3. Fischer, M. H., and Moore, G., *Am. Jour. Physiol.*, 1907, xix, 314.
4. Benedict, S. R., *Jour. Biol. Chem.*, 1908–09, v, 485.
5. Vernon, H. M., *Jour. Physiol.*, 1902, xxviii, 156.

CONTRIBUTION TO THE CULTIVATION OF THE PARASITE OF RABIES.*

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PLATE 19.

Galtier[1] and Pasteur[2] demonstrated the infectiousness of rabies. Negri[3] next described the characteristic inclusions in the ganglion cells, and Babes,[4] J. Koch,[5] Volpino,[6] Proescher,[7] and others found minute granular or pleomorphic particles in the central nervous system of animals infected with rabies. Remlinger,[8] Poor and Steinhardt,[9] Bertarelli and Volpino[10] demonstrated the filterability of the rabic virus; but the nature of the virus itself had remained unknown.

In the early part of 1912 I undertook to cultivate the rabic virus and I have now obtained certain results regarding which a brief report is appended. About fifty series of cultivations were made with the brain or medulla removed aseptically from rabbits, guinea pigs, and dogs infected with "street" virus, "passage" virus, or "fixed" virus. Usually the animals were etherized just before spontaneous death occurred.

The method that has yielded the result to be reported is similar to that employed successfully for the cultivation of the spirochætæ

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[1] Galtier, *Compt. rend. Acad. d. sc.*, 1879, lxxxix, 444.

[2] Pasteur, L., Chamberland, and Roux, *Compt. rend. Acad. d. sc.*, 1881, xcii, 159.

[3] Negri, A., *Ztschr. f. Hyg.*, 1903, xliii, 507; xliv, 520; 1909, lxiii, 421.

[4] Babes, V., Traité de la rage, Paris, 1912; *Ztschr. f. Hyg.*, 1907, lvi, 435.

[5] Koch, J., *Ztschr. f. Hyg.*, 1910, lxvi, 443.

[6] Volpino, G., *Arch. per le sc. med.*, 1904, xxviii, 153.

[7] Proescher, F., *Berl. klin. Wchnschr.*, 1913, l, 633.

[8] Remlinger, M. P., *Ann. de l'Inst. Pasteur*, 1903, xvii, 834; 1904, xviii, 150.

[9] Poor, D. W., and Steinhardt, E., *Jour. Infect. Dis.*, 1913, xii, 202.

[10] Bertarelli, E., and Volpino, G., *Centralbl. f. Bakteriol., 1te Abt., Orig.*, 1904, xxxvii, 51; Bertarelli, E., *idem*, 1905, xxxix, 399.

of relapsing fever.[11] In the cultures very minute granular and somewhat coarser pleomorphic chromatoid bodies arise which on subsequent transplantation reappear in the new cultures through many generations. The same bodies, so far as appearances are concerned, are obtained from "street," "passage," and "fixed" virus. The smallest of these bodies are just on the limit of visibility with Zeiss apochromatic 2 millimeter lens. On four different occasions I observed in the cultures from "passage" and "fixed" virus nucleated round or oval bodies surrounded with membranes totally different from the minuter granular bodies although arising in the cultures in which the latter occurred. Their appearance was sudden and their duration four to five days, when they diminished coincident with an increase in granular bodies. In one of the four instances the larger bodies appeared in a culture prepared from the brain of a rabbit previously inoculated with a culture of "passage" virus which had given rise to the symptoms of rabies. In two of the instances the larger nucleated bodies arose in cultures prepared from "fixed" virus in which Negri bodies were difficult to demonstrate either in sections or in films.

The general morphological characters of the nucleated bodies are shown, magnified 1,100 times in the plate (figures 1 to 13). Along with them are also shown, for comparison, Negri bodies taken from the brain of dogs succumbing to "street" virus (figures 14 to 20). The cultivated nucleated bodies are actively multiplying, by division or budding, and exhibit the appearance not of bacteria but of protozoa. In size they range from about one micron to twelve micra. Under the dark-field microscope, as in the stained preparations, they show definite characters. The center is nuclear and the membrane is distinct and highly refractive. The united individuals in a multiplying mass may be enclosed for a time in a common capsule (?). By inoculating cultures containing the granular, pleomorphic, or nucleated bodies, rabies has been reproduced in dogs, rabbits, and guinea pigs, as shown by the typical symptoms and positive animal inoculations; while the film preparations from the brain of the animals contained always the granular and sometimes the nucleated bodies in large numbers.

[11] Noguchi, H., *Jour. Exper. Med.*, 1912, xvi, 199.

A fuller report on this subject is reserved for a later publication.

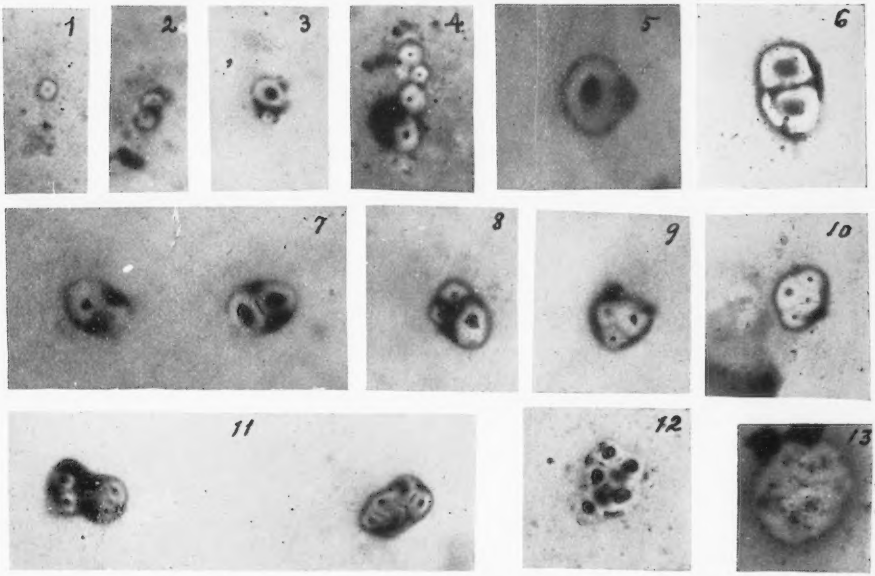
EXPLANATION OF PLATE 19.

All microphotographs were taken from the film preparation stained with Giemsa solution. Magnification uniformly × 1,100.

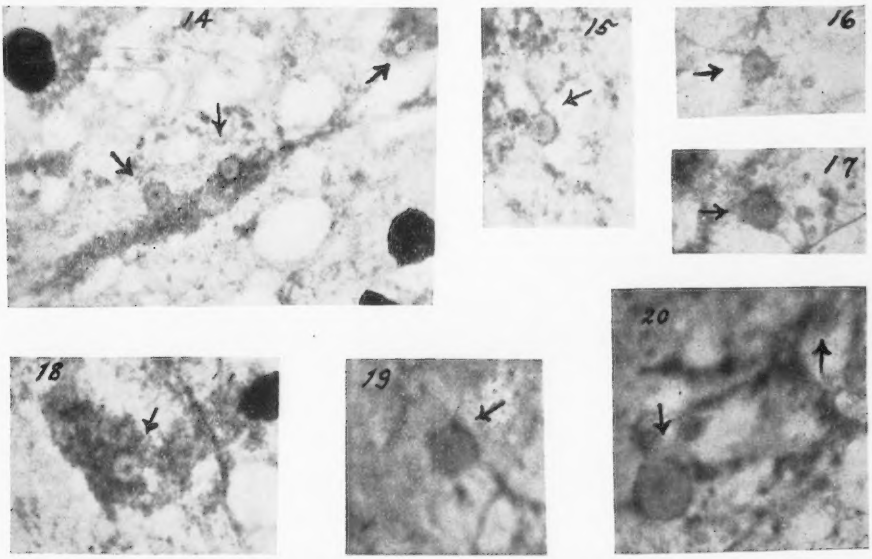
FIGS. 1 to 13. These represent the nucleated bodies of different stages of development in a culture (second and fourth generations, five days old) prepared from the brain of a rabbit experimentally infected with "fixed" virus. In the original material there were no such forms to be seen either in the films or in sections.

FIGS. 14 to 20. These show Negri bodies found in the films prepared from the brain of dogs dying of natural infection with "street" virus. The bodies possess central nuclear substances and distinct cell outlines (membranes). They are usually distributed singly.

A. Nucleated bodies in culture (Giemsa stain).



B. Negri bodies in the brain films (Giemsa stain).



(Noguchi: Cultivation of the Parasite of Rabies.)